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Overripeness of the Egg as a Cause of Twinning and Teratogenesis: *A Review**

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INTRODUCTION

The formal analysis of teratogenesis has become greatly advanced by the insight into the principles of growth and differentiation which was gained through recent progress in experimental embryology and oncology. But, although it was learned that inductors and certain physical and chemical agents are inseparably involved, the actual etiology of pathologic development still remains problematic. While experimental and spontaneous malformations often are strikingly similar, most tools and technics of the researcher are not suggestive of any factors that might plausibly act as initiators of the various forms of congenital anomalies. It has long been known that eggs may suffer damage from unfavorable environmental conditions; but research in this field lagged, because of the obvious uncertainty and complexity of the factors which participate in such reactions. Half a century ago, August Forel coined the term *blastophthoria* as a summary designation for all *blastemic degradations*, which an egg or early embryo may suffer from adverse environmental conditions, poisons, extreme temperatures, lack of oxygen, or overripeness. *Blastophthoria* appears, therefore, distinct from *hereditary degeneration* as brought about by genic mutations. However, it is well to remember that the conditions of the external environment as well as the phenotypic milieus of the cells within an organism sometimes are gene-controlled. Hence, blastophthoria may also originate as an indirect effect of genic factors.

* Besides a discussion of literature this contains also a report on work by the author, done under a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

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A widespread occurrence of blastemic degradation in human reproduction is clearly revealed by the studies of Hertig and Rock (39). They show that, of ovulated eggs, more than half are either not fertilizable—even if contact with sperms seems assured—or result in zygotes of such poor quality that they decompose during the very first days following impregnation. Furthermore, of all the "ova" recovered during the first and second weeks, about one-half exhibit visible abnormality. All considered, one may doubt whether as many as 20 per cent of all ovulations produce "good eggs."

The cause of such low quality of the human eggs may sometimes be hereditary and based on chromosomal aberrations. However, experiments with animal eggs show that as a rule the early stages of development can be passed through quite normally, even if the chromosomal arrangements are grossly upset, provided that the cytoplasmic constituents are normal. The descriptions by Hertig and Rock of the abnormal human cleavage stages and the present writer's own observations on similar occurrences in rodents leave little doubt about the prevalence of *cytoplasmic degradation* in mammalian eggs. The etiology of this condition in mammals will be discussed later. The difficulty of procuring mammalian eggs in large numbers suggests the use of fish and amphibian eggs for the experimental elucidation of the causes and consequences of blastophthoria.

The fact that between normalcy and death of aging eggs there lies a period of progressive morbidity was recognized by Pflüger (69) as early as 1882. Through his and subsequent work by O. Hertwig (40), R. Hertwig (44), Witschi (88–93, 96) and others, it has become evident that the condition of overripeness, as arising in the un-

fertilized frog egg, is the cause of the development of characteristic malformations and also of a surprising shift of the sex ratio in favor of the males. The first part of the present paper reviews the results of the author's former work with *Rana temporaria*, which was only incompletely covered by preliminary and widely scattered publications; furthermore, it contains the report on new investigations that were carried out at Tübingen in the spring and summer of the year 1949,[1] and a few data on work with *Rana pipiens*. The latter will be more fully presented in separate publications. The second part combines a discussion of the teratogenic principles, as far as they may be recognizable in this material, with a survey of the literature on the experimental production of abnormal development in fishes, amphibians, and mammals.

I. EXPERIMENTS WITH FROG EGGS

1. Material and Methods

The eggs of frogs are well suited for experimental purposes because they are released in large numbers—1,000 or more within a few minutes—and are almost invariably in a perfectly healthy and viable condition. It is indeed exceptional to find defective or dead embryos in the naturally deposited spawn of frogs. After artificial insemination there may be losses up to 10 per cent, which are due to imperfections in laboratory technic rather than to original defects of the eggs.

At ovulation the mature frog eggs fall into the open body cavity, but are soon taken up by the oviducts and collected in the paired uteri. The uterine eggs are at the stage of the second meiotic metaphase, the first polar body as a rule having been formed while the egg descended through the oviducts. They are unable to develop any further, unless fertilized. If, during the reproductive season, the weather and especially the water temperature are favorable, ovulation and fertilization may follow each other within a few hours. On the other hand, falling temperatures defer spawning and sometimes cause retention of mature eggs in the uteri for several days. As a rule this causes little harm, since low temperatures tend to preserve the viability of the eggs.

In the laboratory, mature eggs can be obtained by laparotomy or by stripping. Placed in dry Petri dishes, they can be inseminated artificially with suspensions of sperms prepared from testes and seminal vesicles. One can induce female frogs to retain their eggs in the uteri by separation from the males and confinement in dry containers. If such females are kept at room temperature (20° C.), their eggs remain normal only 3–4 days, then become overripe, suffer a gradual decline of vitality, and die at the end of about 1 week. Sometimes the females will deposit the overripe eggs if reunited with males, but usually it is more expedient to secure the eggs by stripping or by laparotomy and to fertilize them by artificial insemination. Since polyspermy occurs much more easily in overripe than in normal eggs it is essential to use not too concentrated sperm suspensions. A quantitative study of the most adequate concentration still has to be made. It is complicated by the extreme variability of the size of the testes, individually, as well as from species to species. For *Rana temporaria* one drop of the fluid taken from the seminal vesicles diluted with 10 ml. of water was found adequate in interspecific hybridizations, in which the danger of hyperspermy is similarly disturbing, as in the work on overripeness.

The breeding season of *Rana temporaria* is a very restricted one, extending scarcely over more than 2 weeks in a given locality and under normal weather conditions. Breeding usually starts when the water temperature rises to 9° C. In temperate regions this occurs as early as February, in the high Alps and the Baltic North only during April. Under laboratory conditions eggs may be obtained prematurely by exposure to the proper higher temperature and through implantation of hypophyses. However, the shift is limited to only about 1 month. Enforced early ovulations give unripe eggs that either cannot be fertilized or develop abnormally. *Rana pipiens* is not as restricted in its reaction to hypophyseal implants; but in early winter it is less responsive to stimulation than toward spring.

2. Abnormalities of Cleavage, Gastrulation, and Neurulation

Even early cleavage stages betray the abnormality of overripe eggs. If an egg was but slightly affected, the third cleavage plane cuts nearer the animal pole than normally, so that at the eight-cell stage the animal quartet of blastomeres is smaller than usual (compare Figs. 1 and 2). Later, as cells become more numerous and smaller, the difference between normal and abnormal morulae may almost disappear. However, at least in the more severely affected cases, a noticeable disproportion in the size of vegetal and animal cells persists, and the individual cells are often more rounded and separated by deeper furrows than normal (Fig. 3). Usually, highly overripe eggs do not develop beyond the morula or early blastula stage. They survive for hours and sometimes for 1 or 2 days without being able to gas-

[1] The cordial hospitality which was enjoyed in the laboratories of Drs. A. Kühn and A. Butenandt at the University of Tübingen is gratefully remembered.

trulate. Often large districts, mostly of the vegetal hemisphere, stop cleaving and form islands of one or several large, multinuclear cells within the more normal parts of the blastulae. Such severely affected germs are of interest because they exhibit clearly two distinctive characteristics of the overripeness effect: the lessened cohesion between the cells and the often regional or spotty distribution of maximal damage. A third generally observed feature is the irregular distribution of the brown pigment; overripe eggs and morulae are of an unhealthy grayish color.

It should also be mentioned that the described deviations from the normal cleavage pattern are distinct from the baroque segmentation which follows polyspermy. In groups of overripe eggs double and multiple insemination is more frequent than in normal cultures; but we may disregard this now, since most such eggs die before gastrulation and thus do not contribute to the teratology of the later stages.

During gastrulation the blastoporal lips obviously encounter great difficulty in executing their epibolic movements over the yolk-filled vegetal cells. As a consequence, the yolk plug is of abnormal size and its invagination is more or less delayed (Figs. 4, 5). In extreme cases, the closure of the blastopore is outrun by the progress of neurulation and the development of the head. As a consequence, the lower spinal cord begins differentiation in halves, separated by the yolk plug (Figs. 5, 6). This is the origin of the frequent malformation called *asyntaxia dorsalis* or, more commonly, *spina bifida* (Figs. 6, 9), which becomes the major cause of the high mortality occurring at the late neurula and early tail-bud stage. Surviving larvae sometimes show extensive duplications of the lower body region, and double tails (Figs. 7, 8). This latter occurrence proves that each blastoporal lip is capable of more than only hemi-embryonic differentiation. In addition to the expected outside halves there form also parts of inner halves through regulative processes which were described by Roux under the name of post-generation.

Another effect of severe overripeness that becomes visible at the neurula stage is the loss of the capacity for histologic differentiation. Since neural differentiation is deeply affected, these malformations are externally recognizable by the deficiencies of neural folds and brains. Their histology will be described in section 5.

3. Duplications

A tendency to produce double monstrosities is one of the most significant traits of overripeness. In spite of its irregular manifestation, this phenomenon assists in the elucidation of the physiologic changes that occur in the egg, when fertilization is delayed.

a. Axial duplication: twins and triplets.—The occurrence of Siamese twins certainly has been often overlooked because the large majority of them suffer from general degradation and die before or shortly after hatching, i.e., as small embryos, 3–7 mm. long. Axial twins may be completely symmetric and of equal size, or they are unequal, permitting distinction between a large autosite and a smaller parasite. All 36 twins of this group show distinct duplication of upper parts and often complete fusion in the lower body and tail regions. This is an indication that at gastrulation there was only one blastopore formed, with two inductive centers in the blastopore lip. In overripe cultures the early blastopore often has the shape of an unusually long and uneven furrow (Fig. 4). There appears to be considerable difficulty in accomplishing gastrulation, and one can repeatedly see the furrow grow deeper and again flatten. Later, the blastopore may be distinctly two-winged, and the yolk plug may show a central depression. Such pictures suggest that the unusual size of the inert yolk mass may be causally related to the differentiation of multiple induction centers. Unfortunately, none of the eggs selected for individual observation happened to be one developing into twins. Nevertheless the described aberrations in the blastoporal region of overripe eggs point to the nature of the factors that are the immediate cause for twinning.

Symmetric twinning in some cases involves mostly the frontal head region, but in others external signs of duplication are noticeable even down to the tip of the tail. Figures 10–14 illustrate the first type. Twins A (Fig. 10) and H (Fig. 14) were preserved when newly hatched (stage 21), while twin B (Figs. 11–13, stage 25–26) had lived for some time as a free-swimming and eating double larva. The younger ones have external gills, the older is provided with gill sacs that open, as in normal larvae, through a spiracle on the left side. There are completely separated oral openings and two pairs each of suckers, noses, and eyes (Figs. 11, 12). On sections one finds two hypophysis glands and a single pair of otic vesicles. The limit of general duplication is marked by the presence of three preotic ganglia, of which the one in the center sends nerves into both the left and the right foreheads. It is smaller than the two lateral ones, consisting mainly of trigeminal elements. The fusion of the neural tubes begins in the diencephalic region but remains incomplete as long as the notochords stay separated. The latter start at some distance from one another in the infundibular re-

gions, gradually approach one another (Fig. 12), and come to lie directly side by side in the lower head or neck region. From here on they run parallel (Fig. 13), at least down into the sacral region (twin A) or even to near the tip of the tail (twin B); both are of the size of a normal chord, and after fusion the single diameter is, for some distance, above the normal size. There exists a peculiar relationship between notochord and floor plate of the neural tube, the latter following closely the surface of the former. As a consequence, in the spinal cord one observes not only a duplication of floor plates but also of adjacent basal plates (Fig. 13), the inner ones fusing in the midplane of the twin body. Separate ventral nerve roots issuing from these inner basal plates connect with the crosswise muscle bundle which is wedged between the notochords. The roof plate of the rhombencephalon is often widely extended, with hydrocephalic conditions varying considerably in severity (Fig. 10 *a*). In comparison with dorsal organs, the ventral ones show less extensive duplications. The oral cavities meet and fuse below the midbrains (Figs. 11, 12). As in normal larvae, there are two thyroid glands, and the entire branchial apparatus, heart, intestine, and respiratory organs are of the single type.

In relatively rare cases, twinning is so extensive that all organ systems are at least partly duplicated. In case H (stage 21) shown in Fig. 14 the upper halves of the twin bodies are not only completely individualized but also widely separated. The common tail has a simple ventral fin but double dorsal fins. The central nervous systems and the notochords are independent except in the final quarter of the tail, where they fuse. The inner muscle segments begin to join at the ninth somite level, that is, near the lower end of the body. From here on one still counts eighteen segments, running between the paired neural tubes and in the triangular space above the notochords. The notochords as well as the neural tubes fuse immediately below the 27th somites. The two hearts are entirely separated. There are four complete pronephric bodies; the ducts of the two lateral ones have grown downward in the normal manner and open into the one, common cloaca. The ducts of the two medial kidneys run mesially rather than caudally; they soon meet and form a largely distended, blind sac, resulting in an externally visible bulge of the body wall. This hydronephrotic condition is proof that the excretory function of the pronephros is initiated even before stage 21. The intestinal complex is still crowded with heavy yolk granules. The stomach regions are independent loops; the livers are connected by some adhesions. The small intestine consists of a simple, large yolk-filled mass from which at more advanced stages a single spiral tube would later have differentiated. The descending aortae fuse in the sacral region into a single caudal artery.

It should be added that the specimen just described is not in every respect symmetric. The right twin is grossly normal and attaches nearly radially to the ventral yolk mass, but the left one is slightly microcephalic and distinctly microphthalmic, without lenses. The attachment of the upper body of the latter to the ventral yolk is rather tangential than radial.

In *asymmetric twins* one conventionally distinguishes between the larger *autosite* and the smaller *parasite*. In our material of twenty pairs and triplets the completeness of the parasite is a function of its size and of its position on the autosite. With diminishing size, but also with increasingly ventral attachment, the cephalic parts of the parasite become defective or entirely absent.

In case Z a small parasite is attached to the right side of the head of the otherwise normal autosite. An externally visible pore is the opening of the nasal pit (Fig. 15, *n*). On sectioning, one finds a single nasal cup, formed by a sensory epithelium of normal thickness and histologic structure. It has no internal opening but is connected by an olfactory nerve with a mass of nervous tissue, obviously the stem of the prosencephalon. The parasite has no eyes; on top of the brain, directly under the skin lies an epiphyseal organ of normal size. The small mesencephalic part is fused to a solid stump representing the upper rhombencephalon, which branches off the same part of the brain of the autosite, below the roots of the acoustic nerve. The autosite has two ears, of which the right one is somewhat defective. Both have nerve attachments to the brain of the autosite. A third ear is located at the right side of the parasite and connects with its rhombencephalon. It is, therefore, an accessory organ, belonging to the parasite (Fig. 16). The pharynx of this larva does not show a definite duplication, but it is laterally bifurcated. There is only one oral opening, and the number of visceral arches is normal on both sides. However, on the right the branchial vestibulum is divided in two parts, of which the upper extends out to the first branchial arch (supplied by the third aortic arch). The lower one forms a sac bounded by the three remaining branchial arches and supplied by the aortic arches four, five, and six. This bifurcation of the pharynx is accompanied by a partial duplication of the heart (Fig. 16). The venous sinus and the atrium are single, but the latter opens into double ventricles, the larger of which supplies the entire left branchial apparatus and also the upper division of the right side, while the smaller supplies the lower three arches of the right side. The two aortic trunks are connected by a vessel that seems to correspond to inner fourth aortic arches (Fig. 16). The peculiar mixed adherence of the three lower branchial arches is also reflected in the innervation. The right postotic ganglion roots partly in the brain of the parasite and partly in that of the autosite. From it issue the branches of the glossopharyngeal and the vagus nerves that supply the three branchial arches. Directly below the ear of the parasite a small vesicle is imbedded in the dorsal body wall, containing a rudimentary glomus and a nephrostome. This accessory pronephric chamber is not connected with the general body cavity.

Case N (stage 21) illustrates the *type of a fairly well developed parasite*, attached at a lower body region (Fig. 18). It is distinctly accessory to the autosite which determines the main axis. Laterally attached parasites become in time more and more forced aside and shift toward the pelvic region (Fig. 20). The very similar though older cases P (Fig. 17*A*) and O (Fig. 21) show that later, at stages 25 and 27 the parasite is partly overgrown and absorbed within the general body contour. Evidently, by the time of metamorphosis the parasite would have been reduced to a relatively inconspicuous dermoid cyst. N, P, and O represent an age series of the same type of twins. Serial sections through N and P show that only the fore- and midbrain region is seriously reduced. The rhombencephalon is fully developed, though somewhat abnormally shaped, due to shortening of the notochord; it is seen obliquely sectioned in Fig. 19, attached to rudiments of fore- and midbrain. The pre- and postotic ganglia are nearly normal in size and shape. Also, the otic vesicles (Fig. 19 shows the left one) and, in the older pair, the otic capsules present the normal aspect. However, the mesencephalon and the prosencephalon consist merely of a very reduced and partly dispersing ganglionic mass. Vestigial olfactory organs in the form of epithelial vesicles are present in

the rostral parts of the parasites (Fig. 19, arrow points to right one). They are connected to the epidermis by cellular strands that still indicate their placodal origin, and with the vestigial forebrain by some nerve fibers; but there are neither external nares nor internal choanae (Fig. 17*A*). The rhombencephalon gradually tapers off into the spinal cord, which fuses with that of the autosite about the middle of the tail. On cross sections of case N the diameters of the neural cord are consistently larger in the parasite than in the autosite. Ganglia in segmental order accompany the cords of parasites as well as of the autosites. The notochords of the parasites are much reduced. They begin only at the level of the fourth somites, and their diameter is not more than one-fourth of the normal. In case N this slender notochord runs down the full length of the parasite and fuses with that of the autosite at the base of the tail, where the spinal cords also merge; in P it ends without fusion (Fig. 17*A*). One observes here a further relationship between the notochord and the general morphology of the neural tube. Where the notochord is reduced in diameter, the floorplate disappears, and the neural canal ventrally is only a very narrow slit; but, where the chorda is fully absent, the ventral part of the canal disappears completely, and only its dorsal part persists. Similar relationships exist in the rhombencephalic region (Fig. 19). The somites start as complete series in the autosites as well as in the parasites. In case N the sixth right muscle segment of the parasite joins and partly fuses with the sixth left of the autosite (Fig. 19, arrow). The union becomes progressively more intimate, and in the tail also the left segments of the parasites flow into the corresponding left ones of the autosite. Both twins (N and P) have four pronephric bodies, each with a glomus. As in the symmetrical twin H, the inner nephric ducts end in a common, widely inflated sac, and ducts as well as kidneys show a hydronephrotic condition. Autosite P has developed mesonephric bodies and a pair of small sex glands. The parasite has only the barest vestiges of a mesonephros. It attracted also some primordial gonia which, however, did not reach a gonad-forming area but lie scattered in the mesentery between the twins (Fig. 17*B*). In P both the parasite and the autosite have formed a pair of arm buds, but only the latter has leg buds. In the older case O, the same arrangement of small legs and arms can be recognized, with toes and fingers clearly differentiated (Fig. 21). Pharynx and visceral arches of the parasites are fully present, though the mandibular arches are reduced in size and the opercular folds of the hyoid arches do not join with the body folds over the heart bulge (Figs. 17*A*, 21). Thus, the gill sac remains a wide-open cavity. The four branchial arches carry tufts of gills and have a cartilaginous skeleton. Since the mouth cleft is very narrow or closed, the gill slits seem to serve for the intake as well as the expulsion of water. In the live animal, extensive respiratory movements of the branchial arches were noticed. The heart lies in a pericardial sac, attached to the body wall of the autosite, above the head of the parasite (Fig. 17*A*). It supplies the branchial arches through four pairs of aortic arches. From the lower pharynx the glottis leads into lung sacs of nearly normal size. Esophagus, stomach, and a short intestinal tube of the parasite bend upward and join the digestive tube of the autosite in the duodenal region. The connected but nearly fully doubled livers have a common bile bladder. Although the stomach and intestine of the autosite are distended with food (feeding started 10 days before preservation of the pair), not a particle seems to have entered the narrowly contracted system of the parasite.

In another group are comprised a number of *cases with small parasites* (Fig. 22), *lacking notochords* (Va, Vb, Vg, Vh, Vl, Vm). The somites form an unpaired median series directly ventral to the spinal cord. The latter is a compact round cord; its cavity either is reduced to a small dorsal canal or is missing; a longitudinal fiber tract runs near its ventral surface. Spinal ganglia, if present, may be more or less distinctly paired, but often form single ventral nodes (Fig. 23). In the uppermost part of the heads one finds a mass of poorly organized head mesenchyme (Figs. 24–26). Many cells are in mitotic division, while others seem to break away, assuming the shape of spindle cells. A third type of cells is small, round, and includes a densely staining nucleus. Possibly, degeneration is imminent in some of these. Imbedded in this mass are ganglion cells, in groups or scattered among the mesenchyme. The uppermost well organized neural structures are ganglia and otocysts. The latter lie close together (Fig. 24) or may even fuse (Fig. 26, case Va). These vesicles are accompanied by acoustic ganglia which connect with the stump of the rhombencephalon or, in its absence, with the spinal cord. In general the central nervous system is histologically poorly differentiated, often retains a blastemic character and sometimes fuses with the similarly undifferentiated muscle blastema (as in Fig. 27). At least, the uppermost three of the normal series of somites are lacking. In case Va (Fig. 26) the series begins with a rudimentary fourth, immediately below the heavy rhombencephalic swelling which may be seen in the photograph. Somites four and five are outside the represented section, which, however, shows the sixth and the seventh, the latter fusing with the seventh of the autosite (arrow in Fig. 26). The endodermal parts of the parasites considered in this group branch from the upper intestinal tract of the autosites. In five, some liver tissue, a coiled stomach, and lung buds have differentiated. The pharynx is even more vestigial than in the preceding group; its reduction progresses in the direction from oral membrane toward the point of bifurcation. Some pouches and rudimentary gills are present in three cases (Figs. 25, 26). In two (Vh, Vl), the intestinal tube ends at the level of the small lung buds (also visible in Fig. 24, left). Accompanying the pharyngeal reduction, the differentiation of all organs becomes very rudimentary, and there is no continuity between the head and the rump-tail parts. The pronephric bodies are fused with one another, and empty through the left pronephros and duct of the autosite. In the first three cases (Va, Vb, Vg), with larger parasites, pronephric ducts have arisen of which the inner one always fuses with the neighboring duct of the autosite. Hydronephrosis develops similarly as in previously described cases.

It is significant that secondary embryo formation always seems to involve the endoderm. In the last case of this series (Vm) the parasite consists only of teratomatous swellings, no longer resembling the organized shape of an embryo. In the upper pronephric region an externally noticeable bulge proves to be an almost solid ectodermal enlargement within the dorsal ramus of the lateral line (Figs. 28, 29, arrows). Below the pronephros a similar though much longer swelling has developed in the medial ramus (Fig. 30, arrows). At this same level of the autosite a strand of loose and entirely undifferentiated endodermal cells rises through the mesentery and, passing around the ventrolateral edge of the somite, connects with various mesodermal condensations that apparently also belong to the parasite (Fig. 30, condensation above pronephric duct). Mainly, however, the endoderm forms a large and thick plate under the dorsal lateral line nerve, reaching even the epidermal surface (Fig. 30, upper arrow). This fusion resembles at the same time the development of pharyngeal

pouches and the formation of cloacal membranes. From the observation that the notochord can be absent, while endodermal participation regularly shows a direct relationship to size and level of organization of the parasites, one may conclude that the primary inductors are located in the prospective endoderm. The only possible exception to this rule is exhibited by case Vb in which a second parasite is present, consisting of a short piece of neural tube and a dorsal tail fin. It is attached to the distal part of the tail of the autosite. A complete series of cross sections shows that individually this piece is not connected with the autosite nor with the first parasite of this triplet monstrosity. However it follows behind the end of the larger parasite and the position of the extra tail fins suggests that this dwarf may have been produced through induction emanating from the larger accessory embryo.

b. Lateral duplication: polymelia and polydactyly.—For any interpretation of the effect of overripeness it is of great importance that, in addition to axial duplication, also polymelia (Fig. 31) and polydactyly may occur. Of the seven specially recorded cases, three had one extra arm, two had two, and another two had three extras each. Multiple accessory arms attach to the same shoulder. In two instances an arm shows signs of partial duplication, resulting in a polydactylous condition (Fig. 31, the most dorsal hand). Polymelia is not a narrowly localized abnormality of shoulder and arms, but involves larger parts of the mesoderm of this general body region, and particularly the sex glands of the same side (90). This may be taken as an indication that prospective gonad cortex and prospective arm bud are neighboring parts of the embryonic somatopleure.

4. Deficiency of Organogenesis

Seemingly contrary to reduplications but nevertheless closely associated with them are the many *reductions* which one observes in embryos from overripe eggs. They originate by partial or complete failure of differentiation, either within restricted areas, or involving virtually the entire embryo. Similar reductions were already met with in the above characterization of twin embryos. However, there is one important difference. The size and the degree of differentiation of twin-parasites are mainly a function of the quantitative potency of the accessory inductor, and even a very small and rudimentary parasite may consist of relatively normal tissues. Quite to the contrary, deficiencies in single embryos parallel rather closely the general degradation of the entire egg, which finds its expression in the failure of histologic differentiation. Temporarily overlooking this factor—which will be treated in the following section 5—the series of microcephalic and acephalic single embryos (Figs. 32–34) nevertheless run a close parallel to those described for the parasite members of twin embryos. For here, too, the reductions start at the frontal end. Sometimes only the hypophyseal placode fails to form (Fig. 35 shows severe reduction); larvae develop that appear normal but for the pale silvery coloration, characteristic of hypophysectomized controls. The next steps of microcephaly are marked by various degrees of microphthalmia, absence of the lenses, synophthalmia, and finally complete anophthalmia (Figs. 36–38). Reduction and absence of the eyes are always associated with a commensurate rudimentary state of the neopallium and the mesencephalic optic lobes. Nevertheless, in some cases of complete anophthalmia one or two olfactory pits and a pineal organ are still present, the former with nerve connections to the rudimentary rhinencephalon. The otic vesicles are never missing if there remains at least one half of the rhombencephalon. Sometimes they are partially fused in the sagittal plane. As the rhombencephalon shortens, the upper end of the notochord recedes. It was found to lie as far down as the level of the third or fourth somite. If the general apical reduction goes further, the entire chord is lacking. Cross sections through chorda-less embryos (Fig. 40) show again a neural tube that has the shape of a round rod. It is either entirely solid or contains a narrow canal in its dorsal or ventral midplane. The spinal ganglia form a single chain between the spinal cord and the unpaired central mass of somitic muscle. The reduction of the cranial parts of the head follows that of the pharynx and the visceral arches. Sucking glands regress and disappear with the reduction of the mandibular arches. Synophthalmic embryos like the type represented in Fig. 38 may still have essentially intact pharyngeal and visceral organs, gills, and a full-sized heart; though the oral parts and suckers are defective or completely absent in every case. On the other hand, the acephalic (achordate) embryos shown in Figs. 33 and 34 have neither pharynx and gills, nor hearts. The upper part of acephalic and microcephalic larvae often consists of a chaotic mixture of rudiments of many organs, neural tissue, visceral cartilages, muscles, and epithelial fragments (Fig. 39). This bears proof that the blastemic mass observed in the frontal part of most microcephalic and acephalic embryos (cf. Figs. 25, 26) derives mainly from dorsal crest material at levels where brain did not differentiate. So far, no embryo of hatching age was found without at least a rudi-

ment of pronephric and of hepatic development. The lower body region and the tail may show various deficiencies, especially abnormality or absence of the notochord and reduction of the fins, but their basic structure remains recognizable except in completely amorphic specimens.

In summary, it is seen that a large class of monstrosities resulting from overripeness may be arranged in a series of apical reductions, beginning at the rostral tip of the head. In addition there occur atrophic conditions of the legs (90), of the sex glands, and of other organs. Their developmental mechanics is of a different nature and must be treated separately.

5. Cellular Pathology and Neoplasia

Duplications and reductions so far were presented merely as anatomic malformations. However, overripeness causes also histologic changes, which are an even more regular and more quantitative expression of the damage suffered by the egg. The change involves a partial loss in the capacity to differentiate. The cells and tissues retain a more embryonic and blastemic appearance than in corresponding normal stages. Such *aplastic effects* may be more or less severe; in extreme cases they lead to disintegration and death of the embryo.

Lethal cases are the already mentioned abnormal morulae or blastulae, which are mere heaps of round cells that prove incapable of gastrulation. However, of particular interest are the less strongly affected ones that still are able to pass through some of the early stages of embryonic differentiation and in this process exhibit various degrees of pathologic changes.

The comparison of the highly overripe twin K (Fig. 43) with the above described H (Fig. 14) shows that twinning and aplasia are separate and to some degree independent expressions of overripeness. On the other hand, our series of micro- and acephali (e.g., M_{41}, M_{50}, M_{54}, M_{59}, A_{28}, A_{20}, A_{53}, and A_{27}) illustrates the close correlation in the progression of acephalism and of aplasia in single embryos.

On sections (Figs. 44, 45), the cells of these embryos appear plump, often round, histologically incompletely differentiated, and exhibit a decreased affinity to basic dyes. Cells from tissues of unlike type and origin have a tendency to intermingle. The spinal tube often is fused with muscle or mesenchyme. The separating membranes are incomplete or entirely missing (Figs. 41, 42). Similarly ill defined are the spinal ganglia. In the head region, ganglionic cell groups are scattered through the globular masses of head mesenchyme (Fig. 46).

Many cells lose their yolk platelets prematurely and instead contain dense clouds of dark brown pigment. It seems adequate to speak here of *metabolic* or *degeneration pigment*. A special study of its formation and later disappearance in the large primordial germ cells is in preparation. Melanoma-like developments are observed in ganglia and generally in cell groups obviously derived from the dorsal neural crest (Fig. 39).

The differentiation of the *vascular system* is deeply affected. The blood-forming cells are large and usually much increased in number; they form thick compact plates (angioblastomas) along the ventrolateral surfaces of the endoderm of hatching embryos (Fig. 49). On the other hand, the development of the endothelial system more often is deficient, and in all cases of advanced aplasia the heart is small or absent (Fig. 44, *h;* in Fig. 39 at *h* one notices a relatively better condition of the muscular than the endothelial heart).

While the growth as well as the differentiation of the affected embryos is generally retarded, there occur also regions of increased, proliferative growth. However, the formation of spreading neoplasms is usually followed by early ulceration, leading to the death of the entire embryo (Fig. 52). Obviously this rapidly fatal course is a consequence of the low cohesion between cells, and of the delicate nature of the embryonic tissues in general. In the following paragraphs it is attempted to characterize shortly the proliferative activity observed in selected systems.

a. Epidermis.—In the epidermis of frog embryos one distinguishes two layers. The thicker surface epithelium consists of cuboid cells that often assume a glandular character, as in the sucking glands and the frontal glands. The latter, it is said, produce proteolytic enzymes aiding the larva in the process of hatching. The surface layer is ephemerous, being completely shed at the time of metamorphosis. The deeper *basal layer* is, at the embryonic stage, a thin, squamous epithelium, usually only one cell deep (Fig. 47). However, in the head region it forms blastemic thickenings, the *placodes*, which contribute to the development of cranial ganglia and sense organs. The basal layer alone is homologous to the epidermis of amniote embryos.

In frog embryos from overripe eggs both layers may start to proliferate, separately or in combination (Fig. 47). The surface layer makes verrucose crusts and papillae (Fig. 45). On the head and over the crest of the rudimentary dorsal fin it often forms thick patches of heavily pigmented glandular cells (Figs. 38, 41, 42). Localized proliferation continues at the larval stage (Figs. 50, 51), when the most peripheral cell layers show a progressive tendency to keratinize (Figs. 53, left side, and 39). Some small papillomas may completely heal, i.e., may be dis-

placed by healthy skin. More often they spread, ulcerate, and if the basal layer is also involved they break open (Figs. 52, 39). Proliferation of the basal layer first leads to the formation of many-cell-deep discs resembling placodes (Fig. 47). When they reach a considerable size, these epidermal epitheliomas usually become invaded by blood vessels that may develop large sinuses and plexuses (Fig. 48).

b. Axial mesenchyme.—It was mentioned before that in microcephalic and acephalic embryos the space above the frontal end of the neural tube is occupied by a mass of mesenchyme, in which are imbedded some rudiments of cranial ganglia (Fig. 46). A similar type of neuro-mesenchyme is formed in the lower parts of the body if the spine fails to differentiate (Fig. 42).

Sometimes one observes also a considerable increase of the axial mesenchyme in the lower body region, between the notochord and the root of the mesentery.

c. Intestinal mesenchyme.—The beginning stages of proliferative growth of the intestinal mesenchyme consist of nodular swellings between mucosa and serosa. Later the mucosa breaks open and the neoplasm begins to shed cells into the intestinal lumen. In the case illustrated in Figs. 53 and 54 the neoplasm has attained a considerable size, extending along the entire right dorsolateral surface of the stomach.

d. Liver.—Proliferations of the endodermal components of the liver probably are quite frequent. They are recognizable as condensations of cuboid cells that fail to organize into trabeculae. The neoplastic cells seem to degenerate early, so that the liver shows no general enlargement.

6. Transplantation Experiments

Since embryonic neoplasms ulcerate early, initiating rapid decay of the entire individual, it was decided to transplant parts of affected embryos into older, free-swimming, normal larvae (Fig. 55). In some cases, the grafts are lost; in others they become established, and their cells ay spread in the host, infiltrating especially the mesenteries (Fig. 56), or grow out along the body walls (Fig. 57). Pigment deposits help to recognize their location. Immediately after metamorphosis, little frogs are difficult to keep alive under laboratory conditions. Therefore, the hosts were killed and the infiltrated parts transplanted into a second host, this time an adult frog. The following outline gives the characteristic history of a successful case.

April 22: Overripe eggs inseminated: examination at the eight-cell stage showed that many eggs cleaved abnormally (culture A).

April 27: Hatching; 199 normal larvae, 87 abnormal embryos, 294 dead embryos (severe overripeness).

April 30: Part of an abnormal embryo implanted into normal larva.

May 21: Second transplantation (from metamorphosed first host into an adult frog).

July 22: Second host was rapidly losing strength; preserved and dissected.

During the last days the body of the second host had started to swell slightly. Upon dissection the body cavity contained some fluid, but mainly it was filled by an enlarged dark brown liver which had formed adhesions with the stomach, the intestines, and also the body wall. Various parts were sectioned. The original implant was still partly attached to the body wall at the site where it had been introduced. It consisted of a fairly well defined mass of cells heavily laden with pigment (Fig. 59). From it issued strands of blastemic tissue that penetrated between the muscles of the body wall (Figs. 58, 60), and also entered some blood vessels. Apparently an attachment to the liver had formed shortly following the operation. From here an inflammatory reaction spread over a large part of the liver. It certainly caused formation of the mentioned adhesions between this organ and the intestinal tract. No focus of infection was discovered that might account for this reaction. However, inflammation seems to be a frequent complication following transplantation operations in adult amphibians. Multiple granulomas of the type shown in Fig. 61 were found in many parts of the body, but especially in the walls of the gut and in the lungs. They often contained heavily pigmented cells and leukocytes and probably belonged in part to the picture of the inflammation. Since new experiments are now in progress, an interpretation of the results of the few preliminary cases will not be attempted at this time.

7. Metabolism of Embryos from Overripe Eggs

The respiratory metabolism of normal eggs and embryos of various species of frogs has been studied by a number of physiologists (11, 61). We have repeated their work and extended it to include also overripe germs (16). Since a separate paper on this phase of our investigations is to appear soon, we need only to summarize the most outstanding features.

After the eggs have left the ovary, and especially while stored in the uterus, they are little or not at all supplied with oxygen. However, in spite of the dwindling rate of oxygen intake, they still produce appreciable amounts of CO_2. The CO_2 obviously cannot be eliminated efficiently and, in some form, accumulates in the eggs. Its excess may eventually depress the rate of decarboxylation. It seems more likely that the effect of overripeness is caused by this CO_2 poisoning than by lack of O_2; for the amphibian egg is able to survive and to develop over a considerable period under anaerobic conditions but suffers soon if held under high CO_2 tensions (see below). If the eggs are removed from the uteri, and especially if they are placed in water, they give off CO_2 in large amounts. Evidently it is derived largely from the accumulated stores, and its release renders the determination of actual metabolism of eggs taken from the uterus a difficult task.

After fertilization three lines of development are to be considered separately: the *normal type*, the *moderately overripe type*

(still able to pass through the larval and metamorphic periods, but showing abnormalities of cleavage and of sexual differentiation), and the *highly overripe type*. The last one exhibits severe teratology and death occurring at various embryonic stages. There appear no essential differences in respiratory metabolism between the second and the first types. Our data agree with those of the above-mentioned investigators, showing that in normal development the respiratory quotient is relatively low during cleavage (R.Q. about 0.75), but rises toward the end of gastrulation and reaches the value R.Q. = 1 during the neurula and early tail-bud stages. Later, it falls again and remains low throughout the entire larval period (Chart 1).

The situation changes markedly in the highly overripe type. The early cleavage stages still follow the normal course. But later the quotient becomes abnormally high in all morulae and blastulae that are unable to gastrulate. A similar and even more pronounced rise, sometimes exceeding R.Q. = 2, is observed at later stages in all embryos that are visibly affected by deficient differentiation (cf. sections 4 and 5, pp. 768–70). The high R.Q., as was shown by the classical investigations of Warburg, is the outstanding chemical characteristic of cancer cells. Even though it is observed not only in cancer but also in otherwise damaged tissues (23), it lends weight to the contention that overripeness leads to cancerous degradation.

A few tests which were made by placing uterine eggs in an atmosphere of pure N_2 showed them still normally fertilizable after exposures of 4 hours. It remains to be determined how long each treatment might be extended. This experiment, proving that the unfertilized egg is quite insensitive to complete lack of oxygen, gains significance in connection with extensive studies on the teratogenic effects of CO_2 (Wenk and Witschi, unpublished results). Over 15,000 eggs were used, of which about one-half served as controls. The results are summarized in Tables 1 and 2. The data of the first one give proof that CO_2 is very harmful, especially if administered in the form of the pure gas (at normal atmospheric pressure). The proportion and types of monstrosities appearing in these cultures are very similar to those above described for the overripe series. Only cultures from eggs treated with 20 per cent and 30 per cent CO_2 were raised to metamorphosis, and in these no deviating sex ratios or gonadal deficiencies were observed. At first sight Table 2 seems to suggest that CO_2 affects overripe eggs less than normal ones. However, if one relates the increase of damage in every culture to the number of normal eggs only, the differences become quite meaningless.

In a final experiment, for 30 minutes pure CO_2 instead of air was filtered through the water in which some 600 eggs had just reached the 8-cell stage. At the end of the treatment the pH of the water had fallen to 6.5, a slight degree of acidity which in itself cannot be responsible for the observed effects on the eggs. In the control series there were 33 per cent of dead or crippled embryos, indicating that the used eggs were overripe. In the CO_2 series, development stopped temporarily following the treatment and was then resumed, but most germs died during gastrulation. Only two larvae hatched, so that the loss was close to 100 per cent. This experiment, complicated as it is with overripeness, needs to be repeated and extended to various stages before a definite conclusion can be drawn. The survey of the entire series of oxygen deprivation and CO_2 treatments discloses a high susceptibility to CO_2 of the unfertilized and the cleaving eggs which, on the other hand, are able to develop without oxygen.

8. Frequency of Manifestations

The above descriptions are based on material from 25 cultures of *Rana temporaria* of different genetic origin and various degrees of overripeness. The same effects of delayed fertilization have been observed in other frogs, but the research reported here deals almost entirely with studies on the one species. Our cultures were raised to metamorphosis, or a few months beyond. They were of different size, consisting of from 100 to 1,500 individuals. Most had been started in connection with investigations on sex differentiation and as a rule not much attention was paid to the early embryonic stages. Hence, it is certain that a number of interesting malformations remained unrecognized and figure only in the mortality records. Many groups were discontinued when high mortalities rendered them unsuited for the purposes just mentioned. *Mortality* is, as a rule, a characteristic measure of the degree of overripeness. It is always higher than in normal sets of eggs and, of course, reaches 100 per cent if the eggs are left in the uteri

TABLE 1

EXPOSURE OF UNFERTILIZED EGGS OF *Rana temporaria* TO AIR-CO_2 MIXTURES IN MOIST CHAMBERS

Duration 35 minutes in the case of pure CO_2; 4–5 hours in all other groups. Lots of 100 or more eggs were used in each exposure. Effects expressed as percentage of *dead + crippled embryos* at time of hatching.

Per cent of CO_2	0.0	10	20	30	50	70	100
Per cent injury	5	6	10	11	27	31	88

TABLE 2

SUMMATION OF EFFECTS OF OVERRIPENESS AND CO_2 TREATMENT

Eggs of different degrees of ripeness from four females (A–D), in lots of from 281 to 830 were exposed to pure CO_2 during 15 minutes, followed by air, 10 minutes. After this they were fertilized with sperm from one single male (m). The per cent of injury (dead + crippled) in the second column indicates the degree of overripeness (increasing from Bm to Dm; Am is normal).

Series ♀×♂	Untreated lots (per cent injury)	CO_2-treated lots (per cent injury)	Increase of injury
Am	2	58	+56
Bm	21	54	+33
Cm	37	53	+16
Dm	79	88	+ 9

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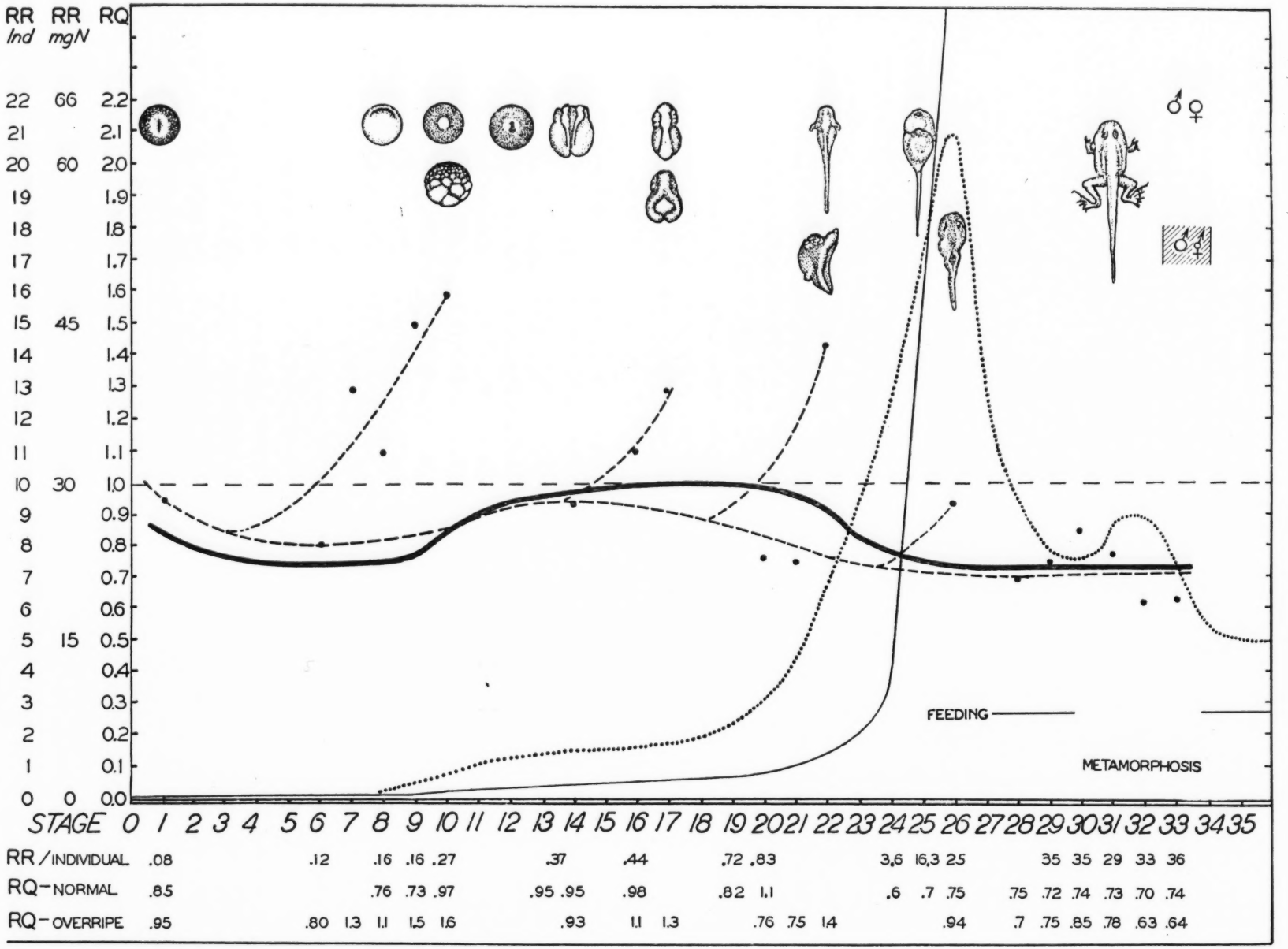

CHART 1.—Respiratory metabolism of developing frogs. *Abscissa:* developmental stages from fertilization to completion of metamorphosis. *Ordinates: RR (ind)*–respiratory rate per individual in normal development (light line); *RR (mg N)*–respiratory rate per milligram nitrogen in normal development; data by I. Wills (dotted line). *R.Q.*–respiratory quotients in normal development (heavy line) and in the development of eggs damaged by overripeness (broken lines). Scattered dots indicate values obtained in the overripe series. At top of the graph small figures illustrate normal and abnormal stages conforming with numerical designations on the base line.

over excessively long times. My own observations as well as those of other workers in this field (13, 44, 100) indicate that overripeness does not ever reach the same degree in all eggs taken simultaneously from a uterus. At hatching, the cultures usually consist of a fairly high number of dead eggs and embryos, varying numbers of "cripples," i.e., malformed embryos, and 50 per cent or more apparently normal larvae with external gills. The above-mentioned (p. 770) culture A with over 50 per cent mortality at hatching time was highly overripe. Not well suited for statistical studies on sex determination, it was used in experiments on transplantation of neoplastic tissues.

The mortality rate depends strongly on supporting factors, on aeration, and especially on *temperature*. In this regard, overripe cultures contrast strikingly with normal controls. In the latter, mortality is equally low at all temperatures, ranging from 10° C. to 22° C. (culture B, Table 3). On the other hand, overripe series show a drop in mortality paralleling the decrease in temperature. This is illustrated by the two cultures D and E (Table 4) and more detailed by the temperature series C

TABLE 3

TEMPERATURE AND MORTALITY RATES IN A NORMAL CULTURE (B) AND AN OVERRIPE CULTURE (C) OF *Rana temporaria*

Temperature (° C.)	Normal culture B		Overripe culture C	
	No. eggs	Mortality (per cent)	No. eggs	Mortality (per cent)
10	155	1.5	372	12
15	337	0.3	434	18
20	320	3.0	456	24
22	281	1.3	459	45

TABLE 4

PARALLEL INCREASE OF TEMPERATURE AND MORTALITY RATES IN TWO ADDITIONAL OVERRIPE CULTURES (D AND E)

Temperature (° C.)	Overripe culture D		Overripe culture E	
	No. eggs	Mortality (per cent)	No. eggs	Mortality (per cent)
10	313	11	503	34
20	240	71	510	90

(Table 3). In the latter experiment, over half of the eggs (55 per cent) developed into hatching larvae at all temperatures. Yet, the rates varied and only 12 per cent died in the cold. Obviously, a particular interest is attached to the one-third which are able to complete embryogenesis in cold water but die if kept near the upper limit of the range tolerated by normal eggs. One can see that low temperature mitigates the damage of overripeness, which in this class evidently has not yet reached the condition of irreversible lethality.

The mortality rate grows rapidly with the *length of the delay of fertilization*. In Table 5 are listed five females whose eggs were taken from the uteri at intervals of from 1 to 3 days. With the exception of case J, different males were used for the first and second fertilizations. In our extensive experiments on serial breeding in *R. temporaria* indications were never found of viability-lowering factors carried by the sperm. The regularly observed increase in mortality in the cases G, H, and I can only be the expression of progressive overripeness. Moreover, in the last case (J) where the same male was used for both inseminations, the result is confirmatory. In the control series F the course seems reversed; but this is due to incomplete insemination of the first batch of eggs, with an unusually high number of eggs remaining uncleaved, probably because of immaturity. In all other groups the highest mortality rate is at the later morula stage (inability to gastrulate; Chart 1) and reaches a second peak at the early tail-bud stage. Of those living at hatching time (stage 21–22), some are teratologic embryos of the types described above. The cultures K–N (Table 6) show

TABLE 5

FERTILIZATION AT TIMED INTERVALS OF EGGS FROM FIVE FEMALES

Both batches of eggs from female F developed normally. All other batches exhibited signs of overripeness, the second inseminations more than the first. In the last column 0 signifies absence of microscopic indications of overripeness; + lowest degree and ++++ highest degree of damage to sex glands.

Culture	Maturity	Interval	No. eggs	Mortality (per cent)	Sex glands
F	normal*		140	21	0
	"	2 days	494	5	0
G	overripe		356	31	+
	"	2 days	810	49	++
H	"		605	16	++
	"	2 days	360	89	++++
I	"		640	34	+++
	"	1 day	339	51	++++
J	"		593	24	+
	"	3 days	345	58	++++

* Possibly some eggs were still immature.

TABLE 6

RELATIVE FREQUENCY OF TERATOLOGIC EMBRYOS AT HATCHING TIME, IN FOUR CULTURES DERIVED FROM EGGS OF A PROGRESSIVE SERIES OF OVERRIPENESS

Culture	Living embryos		Dead eggs and embryos
	Normal	Abnormal	
K	650	35 (5 per cent)	150 (18 per cent)
L	230	50 (18 per cent)	60 (18 per cent)
M	415	107 (20 per cent)	550 (51 per cent)
N	25	15 (38 per cent)	320 (89 per cent)

the approximate range of the *frequency of malformations* in overripe series, raised at temperatures between 18° and 20° C. While it rises with the severity of overripeness (expressed in mortality indices), normal larvae do not disappear except in very extreme cases, with over 90 per cent mortality. The unevenness of the degradation process still remains unexplained. Richard Hertwig tried to separate eggs from different regions of the uterus, in an attempt to elucidate this phenomenon. Our experiments, intended to determine the possible effect of peripheral or central location within the uterus, so far remain equally inconclusive (cf. p. 783).

Very irregular is the manifestation of duplications, *twinning and polymelia*. Unfortunately the records on these malformations are incomplete, because the majority of actual cases must have remained unrecognized. Supernumerary arms and legs are difficult to see and in fact never were recorded before the metamorphosis stage. By this time the affected individuals naturally have become more reduced by mortality than the normal sister-larvae. Of axial duplications the relatively rare symmetric double heads are most readily noticed. With few exceptions, the

twins of all types die at the transition from the embryonic to the larval stages, when they measure only a few millimeters in length.

Nevertheless, the fact that the 34 twins and nine polymelic frogs on record all were found in seven of the total of 25 overripe cultures, while not a single case was ever found in over 100 normal cultures, is sufficient proof that the blastophthoric effect of overripeness enters as the basic factor in the determinative complex. Obviously, there must be other, sustaining factors. The possibility that special genic conditions might favor the formation of duplications will be discussed. Of experimentally analyzed conditions of milieu, high temperature was most effective in increasing the production of duplications. Five of the nine polymelic frogs arose in a group of twenty metamorphosing survivors of a "heat" series (28°–30° C.) of the overripe culture C. Among 548 frogs of the sister series kept at 10°, 15°, and 20° C., there occurred not a single excess limb.

In continental races of the brown frog, the *reduced size of animal blastomeres* and other abnormalities of the cleavage pattern are a rather sensitive indicator of the degree and spread of overripeness in any batch of eggs. In eggs of six females from the British Isles (Sheffield), the diminution of the animal blastomeres was not equally distinct, in spite of advanced stages of overripeness attained in some cases. Possibly the basic conditions of viscosity are somewhat different in eggs of this race. On the other hand, general irregularities and especially the change of pigment distribution were fully as marked as in the continental races.

A very delicate reaction to overripeness is the *dystrophy of sex glands and germ cells*. The latter show precocious disappearance of the yolk platelets, accumulation of pigment granules, and depressed mitotic activity. At the time of metamorphosis the gonads are undersized, slender, and not always clearly differentiated in either the ovarial or the testicular sense. Before or immediately following metamorphosis the small ovaries transform into testes. In the last column of Table 5 the condition of the sex glands of the survivors of each series has been indicated. It appears from this record that seemingly still normal eggs, which developed into "normal" embryos, nevertheless were also affected by the prolonged sojourn in the uterus.

II. DISCUSSION

1. Literature on Overripeness of Vertebrate Eggs

In laboratory work with frogs overripeness is so easily produced, even unintentionally, that its deleterious effect on the eggs was early noticed. Probably Pflüger (69) was the first to devote a special paper to one phase of the reaction complex, the striking reduction of the sex glands. He suspected the shift of the sexual differentiation in the testicular direction, which was later firmly established through experiments by Richard Hertwig (literature to be reviewed in a forthcoming publication). Born found that overripeness favors polyspermy and introduced the term *Barockfurchung* for the abnormal multiple-cleavage type of such eggs. Roux and O. Hertwig made extensive observations on certain phases of the development of overripe eggs. The work of the latter (40) deals mostly with the abnormalities in the gastrulation process that lead to delayed invagination of the yolk plug and spina bifida (p. 764). He wrongly assumed polyspermy to be the major direct cause of the abnormal development, though at the same time he was well aware that the degradation of the protoplasmic cell body also enters as a teratogenic factor. The reduction of the size of the animal blastomeres in overripe but obviously not polysperm eggs (Figs. 2, 3) suggests that yolk and other heavy cell inclusions settle more toward the animal pole than normally. This causes the formation of the large and relatively inert yolk plug which becomes an obstacle to the progress of both invagination and epibolic growth (Figs. 4–6). Gastrulation assumes the aspect of the teleost type, except that in the cases of great delay, the differentiation processes in the blastoporal lips outrun concrescence.

In 1941, Briggs (13) and independently also Zimmerman and Rugh (100) reported on experiments with *Rana pipiens*. The latter kept the egg-producing frogs at 10° C. and cultivated the fertilized eggs at 13° C. Overripeness effects became distinct after the seventh day of retention in the uteri. They became progressively more severe until, at 12 days, all eggs were unfertilizable. The description of developmental stages is brief and incomplete, but gives the impression that the abnormalities are similar to those in *R. temporaria*. Some differences, and particularly the absence of duplications, are probably due to the low temperatures at which these experiments were carried out. Briggs, working also with *pipiens*, kept the ovulating females, as well as the resulting cultures, at about 20° C. Effects of overripeness became evident after uterine retention of 3 days. After a delay of 5 days, 87 per cent of the eggs were still fertilizable, but cleavage was abnormal and the entire group died before the gastrula stage. Contrary to Zimmerman and Rugh, Briggs found that "partial twinning, secondary embryos, and accessory appendages occur in as many as 20 per cent of some overripe cultures." At the time of the first three cleavages, Briggs separated 227 normal from 173 abnormally dividing eggs. Among the latter must have been practically all the polyspermic eggs. This group died off rapidly, and only two tadpoles survived. The group with the normal cleavage type (but overripe from 3 to 5 days) gave 81 normal and 23 abnormal tadpoles. About half of the regularly cleaving, presumably monospermic eggs developed abnormalities "typical for overripe eggs, including tumor-like growths." This

clearly limits polyspermy as a teratogenic factor. A. Brachet (10) and Herlant (38) had shown previously that frog eggs, if penetrated by more than one sperm, seldom complete gastrulation or reach the tadpole stage. Briggs's observations definitely prove that eggs which are doubly handicapped by overripeness and polyspermy succumb even more readily and therefore do not essentially contribute to the picture of teratology after the cleavage stage.

Briggs's work on *Rana pipiens* furnishes evidence that overripeness produces the same effects in this species as in *R. temporaria*. Our own experiments with *R. esculenta*, *R. sylvatica*, and *Bufo americanus* make it clear that delayed fertilization acts as a teratogenic factor throughout the order of the anuran amphibians.

It has often been suggested that malformations like cyclopia and twinning, which are so frequently observed in trout hatcheries, result from overripeness of the eggs. The conditions and practices of artificial insemination must indeed lead to delayed insemination of the eggs of some of the *fish*. To clear up this situation, Mrsic (60) made experiments with the rainbow trout. Kept at about 12° C., the mother fish delivered normally developing eggs until the end of the first week (mortality below 10 per cent). At 2 and 3 weeks the mortality rose to 30 and 54 per cent. Parallel with the mortality grew the percentage of cripples, of which some showed eye defects and double tails. No histologic study of the malformations other than those of the sex glands was reported. Transformation of ovaries into testes was observed in several instances. Werber (85, 86), treating fertilized Fundulus eggs with solutions of butyric acid or acetone, obtained, among other monstrosities, a number of twins that ranged "from apparently symmetrical, well-formed double embryos through various degrees of deformation to highly defective and grossly malformed double monsters." Werber is inclined to consider the age condition of the egg as an important contributing factor in the production of twins and monstrosities, stating that "younger eggs" are "less susceptible" to the chemical treatments than "older" ones.

After the artificial insemination of 100 eggs of the lamprey at least 3 days overripe, Bataillon (6) observed "spontaneous blastotomy" in 40 per cent of the lot. The eggs must have been highly overripe, and the mortality was correspondingly high. Four of the eggs that had separated along the first cleavage furrow produced one pair of hatching twin larvae each.

The teratogenic effect of overripeness in mammals remains almost unexplored. Yet conditions which may bring about germ-cell degradation of the type that we studied in amphibians are obviously widespread. Tubal eggs deteriorate rapidly. In unmated rats, they begin to fragment and decompose less than 2 days after ovulation (70, 97). In the rabbit they become unfertilizable 6 hours (about 8 hours according to Pincus) after ovulation (35), and in the guinea pig 20 hours after ovulation (8). Concerning the guinea pig it was also established that deterioration of the egg begins after the eighth hour.

While thus in mammals delayed fertilization is recognizable as a possible factor of teratogenesis, germ-cell degradation before ovulation is probably of even greater importance. Of about 5 million germ cells that enter the ovocyte stage in the ovaries of a human female fetus, at the most only 400 or 500 may ever be ovulated. All others degenerate within the ovaries before the menopause. Many disappear as primary ovocytes; others, by so-called follicular atresia at later stages. In the ovaries of rats, rabbits, and other mammals one usually finds, at the end of the ovulatory period, several large follicles that have retained their egg. Often one observes a polar spindle, or fragmentation of the egg. Evidently many follicles that succumb to atresia were close to the proper condition for ovulation. It may therefore be concluded that those actually accomplishing ovulation release eggs which are not all at exactly the same stage of cytoplasmic maturity. This assumption is fully sustained by the fact that in all carefully investigated polyovular mammals at least one-third of ovulated eggs either are not fertilizable or produce grossly abnormal embryos (37, 62, 70). The recent investigations of Hertig and Rock (41) prove that the proportion of "bad eggs" is equally high in the monovular human species. Corner (19) as well as Marshall and Hammond (58) studied early fetal death in the pig and the rabbit; they came to the conclusion that the major lethal factor must be sought in the embryo or fetus and not in conditions of the uterine environment. Hammond (35) has further shown that *high rate of fetal atrophy* is an inheritable character in certain breeds of rabbits; but the rate depends on the zygotic condition of the mother, not of the fetus. In combination with the first statement this suggests to us that mother rabbits of a high-atrophy strain must ovulate relatively large numbers of eggs of defective plasmatic structure. If this conclusion is valid in principle, some cases of human hereditary pathology may possibly also trace back to genic factors that upset the mechanisms which normally control the presence and the selection of properly matured follicles at the time of ovulation.

2. The Mechanics of Twinning

It is evident that the actual development of twin embryos must be preceded by the formation of twin centers of germinal organization. Impressed by their discovery that ordinarily the penetration of the egg by a single sperm initiates the embryogenic process, Fol and O. Hertwig (40) forwarded the theory that polyspermy is the cause of twinning and polyembryony. The implied assumption that the sperm essentially contributes to the determination of the embryonic axis and the formation of the blastopore has since been proved erroneous. The subsequent experimental analysis of the first steps of development by a great number of investigators led to the realization that not only polarity but also bilaterality and other spatial relationships are characteristic attributes of the structure of the amphibian egg, even before fertilization (36). Moreover, it was shown that double fertilization does not always prevent the formation of single embryos (38). The persistence of unitary organization in spite of double fertilization is most strikingly expressed in cases of mosaic development, such as those described by Goldschmidt and Katsuki (43) and by Rothenbuehler *et al.* (74). Any causal relationship between polyspermy and twinning is therefore definitely ruled out, and their increased frequencies are independent consequences of overripeness.

Of fundamental importance for the explanation of twinning became a paper published by Driesch in 1891, reporting that blastomeres of the sea urchin, separated at the two-cell stage, had developed each into a complete larva, while incomplete separation led to the formation of conjoint twins. Similar separation experiments with amphibian eggs by Spemann and his school proved that at the two-cell stage a center of organization is so located that it is evenly divided by the sagittal plane. It lies somewhat dorsal to the frontal plane and if, by constriction at the four-cell stage, one separates dorsal and ventral halves, only the former produces an embryo. The ventral half goes on cleaving, yet is unable to gastrulate. If the center is cut in unequal parts by an oblique separation plane, the more ventral half may only incompletely gastrulate and forms a defective embryo. With essentially the same results, morulae, blastulae, and early gastrulae may be cut in two. At gastrulation the center is localized in the dorsal blastopore lip. In spite of strenuous efforts by embryologists and chemists to define the "organizer" in chemical or in structural terms, no understanding of its true nature has been gained as yet. However, some further light is shed on the process of twinning by two lines of investigation which started with discoveries by Schultze and by Holtfreter.

In 1894 O. Schultze (77) induced twinning in frog eggs without separation of blastomeres. At the two-cell stage, the eggs were compressed between glass plates and turned upside down. Rearrangements of the cell components follow this upset of gravitational conditions. In general the heavy yolk sinks from the vegetal toward the animal pole, dividing the archiplasm and displacing it laterally left and right of the first cleavage plane. In at least a number of cases the yolk mass must break up the organizer and thus lead to double gastrulation. In others a segregation may not take place, but the organizer comes to lie between the two separate concentrations of archiplasm, each of which is competent of embryo formation. Thus internal dislocations result in the production of double monsters. Pasteels (64, 65), who confirmed and greatly extended this work of Schultze, interprets the facts on the basis of a double-gradient concept rather than the original inductor theory. However, the principle of twinning through rearrangement of cytoplasmic constituents is common to both interpretations, and the actual differences in viewpoints are without bearing on the problem discussed here. Since in overripe eggs the viscosity conditions are so altered that the movements of the yolk and pigment granules are noticeably atypical, it is conceivable that the organizing material may become divided more readily than in normal eggs. It should not be forgotten that during a period following fertilization and extending into the early cleavage stages essential materials are being rearranged within the egg. The formation of the gray crescent is a visible expression of some of the occurring displacements. It seems that in the ovocyte the prospective induction center lies deep in the cytoplasm and that after fertilization it moves toward the dorsal surface. The flow of the supporting materials may well be affected by the conditions of overripeness. The similarity of some of the twins obtained in our experiments with the types illustrated by Schultze suggests that the increased sedimentation rate, playing together with the generally observed lessened resistance of overripe eggs against deformation by pressure, may occasionally lead to a division of the organizing center.

This principle of *physiologic fragmentation* of the egg—as it may be called in contradistinction to the mechanical division practiced in the experiments of Driesch and of Spemann—was originally believed to be of prime importance for the explanation of twinning after overripeness (88, 89). However, later it was extended by the concept of the formation of *accessory organizers* (93).

The notion that secondary organizing centers may arise anew and independent of the original one was evolved on the basis of Holtfreter's (47) discovery that nonorganizer material of the blastula gains inductive capacity if it is injured or killed by freezing, boiling, or dehydration. Apparently the inductive agent is generally distributed in every cell, though in healthy normal blastulae it is inactivated except in the organizer region. Ideas recently developed by Pasteels (68) carry even further. Repeating and reinterpreting centrifugation experiments of earlier workers, he comes to the conclusion that ordinary ventral ectoblast can be so altered that it may form neural and mesodermal structures by self-differentiation. Obviously, there is good reason to consider also the possibility that the disintegrating effect of overripeness can activate some organizing substances outside the primary inductor region. This would help to explain the widely established fact that monovular twins are afflicted with other abnormalities more often than single individuals. Of the 36 frog twins which served for this study only two are free of gross defects. In the large majority the autosite as well as the parasite exhibits various forms and degrees of teratologic development, and duplications are more frequent in the highly damaged than the moderately overripe class. Notwithstanding this unmistakable relationship, cytoplasmic degradation alone seems barely sufficient to explain the appearance of definite new centers of organization. Probably axial duplications are a resultant of several factors, of which the following appear to be of foremost importance: (*a*) Overripeness tends to promote physiologic fragmentation and to activate latent inductor capacity; (*b*) altered conditions of viscosity render the structure of the overripe egg more than normally susceptible to pressure and gravitational disturbances; (*c*) the large size of the yolk plug together with the lessened cohesion and physiologic integration between cells (cf. following paragraph) favor the establishment of multiple inductive centers in the enlarged blastoporal region.

Polymelia, the second type of excess formation, presents the problem of the origin of multiple induction centers in a new version. It has often been shown that mechanical division of preprimordia or of early buds of forelimbs leads to the development of at least one arm from each fragment. This, of course, parallels the separation experiments with cleaving eggs and blastulae. But the case of spontaneous polymelia bears in itself evidence that all interpretations of duplication phenomena that rely strongly on yolk gradients or on the displacement of yolk masses are not universally applicable. At the time when the multiplicity of inductive centers becomes established, there is little if any yolk left in the limb-forming cells. The loss of unity of organization probably is more of the nature of Bataillon's blastotomy (cf. p. 775). So far, no premetamorphic stages have come under observation. However, the study of Bonnevie (9) on the development of polydactyly in the Bagg-Little strain of mice (5, 57) carrying the gene "bleb" is highly suggestive; for blebs, i.e., subepidermal, fluid-filled vesicles, often appear also in tail-bud embryos from overripe frog eggs. Bonnevie failed to clear up completely the relationship between the development of blebs and of extra toes. According to her presentation, the two morphologic manifestations appear nearly simultaneously. However, the location of the vesicles rather suggests that the liquefaction of intercellular substances, which must precede the formation of blebs, may be the original cause of the duplications.

The relative unimportance of the yolk as a morphogenetic agent is also indicated by the fact that twinning and polymelia occur similarly in birds, mammals and amphibians. Neither the total amount of yolk, nor its distribution within the eggs or the blastomeres shows a direct relationship to the frequency of excess formations.

Willis (87) in his excellent review opposes the idea that teratomas may originate by a process of twinning. However, he admits that "the site of distribution of teratomas points to the operation of growth disturbances emanating from the primary axis—the notochord and contiguous structures which are derived by invagination of tissue at Hensen's node in the early embryo and which constitute the primary organizer." If allowance is made for some differences in terminology, these statements only affirm that certain forms of mammalian teratomas are closely related to the types of amphibian malformations obtained in our experimental work.

3. Causes of Hypoplastic Development

Some of the various forms of deficient differentiation which are observed in the development of overripe eggs may also result from physical or chemical changes in the environment or within the egg and the embryo.

a. Abnormal cleavage.—Abnormal cleavage may be caused by either nuclear or cytoplasmic impairment. X-rays as well as certain chemicals injure specifically the nuclei, mitotic spindles, and chromosomes. Extensive studies were made of the effects of colchicine (51), urethan (80), and estradiol (82). It is noteworthy that estriol and estradiol cause extensive karyorrhexis if added to the water

in which the cleaving eggs are submerged, while estrone, in local application, was shown to be a highly effective organizer substance (83). Such findings lend support to the contention that estrone and the other estrogens may not themselves be organizer substances, but by their injurious actions set free inductive potencies from ordinarily inactive cells. In later development, germs partially damaged by estradiol may produce hemiembryos and other mosaics of territories, exhibiting various degrees of abnormality, which in some instances seem to correspond to alterations of nuclear composition suffered by individual blastomeres. Testosterone is much less injurious during the cleavage stages but interferes with the normal progress of neurulation. Töndury (81) finds no correlation between the damage to the mitotic process and the formation of neural folds.

In overripe germs, as long as they are not yet disintegrating, mitotic defects or chromatin elimination have not been observed. The damage obviously is cytoplasmic in nature. Therefore, it is not surprising that the characteristic aberration of the cleavage stage, namely the relative size reduction of the animal blastomeres, is closely imitated by simple *physical* means affecting the structure of the cytoplasm. A large number of investigators have reported that centrifugation, as well as the application of heat, result in the formation of small polar blastomeres and may cause cleavage to proceed according to the meroblastic type (42, 43). Furthermore, Bataillon (6) and Montalenti (59) have shown that the separation of the blastomeres in the lamprey may most conveniently be obtained by temporary (4 hours) transfer of the fertilized eggs into sugar solutions (10 to 15 per cent), i.e., by an osmotic factor rather than by overripeness.

b. Delayed gastrulation, protruding yolk plug, and spina bifida.—These abnormalities occur frequently after centrifugation of uncleaved eggs (43, 63, 66) or after exposure of the germs to immediately sublethal degrees of heat (42, 45). Similar effects were also obtained by the admixture of a growth inhibitor substance, hexenolactone, to the aquarium water in the amount of 5 mg/l. Briggs (14) furnished proof that this substance is primarily affecting the cytoplasm of the egg.

In the chick, Ancel (1) produces spina bifida by incubating the eggs at 40.5° C. About 8 per cent of the surviving chicks show this malformation which appears only very rarely in controls at 37.5° C. Spina bifida was even more frequently obtained by placing on the normally incubating embryo at the stage of eight somites (26 hours) a drop of distilled water (16 per cent malformations) or of solutions of various chemical substances (23–30 per cent malformations). It should be mentioned that the term spina bifida carries somewhat different connotations in its applications to amphibians and to amniotes. In the former it refers to nonfusion of blastoporal lips; the entire neural cord is split and in typical cases also the notochord (cf. p. 765). In birds and mammals the same term signifies only the dorsal nonclosure of the neural tube or also a mere fusion defect of the dorsal vertebral arches. The two are closely related, embryologically, but the amphibian type originates as a gastrulation defect, the amniote type as a disturbance of postgastrulation. It probably would have saved some misunderstandings if Hertwig would have adopted Roux's term of *medullary asyntaxia* for the amphibian type.

c. Microcephaly, acephaly, and failure of notochord formation.—These characteristic malformations of the overripeness cultures may likewise be obtained by centrifugation of uncleaved eggs (66) and by cultivation of the germs at high temperatures (45). Moreover, similar series of hypomorphoses result from the treatment of blastulae and gastrulae with lithium chloride solutions (54, 67). Somewhat less extensive are the disturbances that follow the application of a large variety of other chemical substances, salts, and organic compounds (21, 41, 48). In some of the treatments chemical and osmotic factors seem to combine in the determination of the abnormalities. Several authors make reference to the peculiarity that the reductions form a rostro-caudal series, beginning with defects at the tip of the head and ending with acephaly, absence of notochord, and general abnormalities of the upper thoracic region. Various interpretations of this phenomenon have been offered. Werber (85), who produced similar monsters in teleosts by submersion in solutions of butyric acid and acetone, proposed a theory combining chemical and osmotic blastolysis with the concept of metabolic gradients in the sense of Child. Later, Wright (99) followed related lines of thought in his attempt to interpret hereditary anencephaly and polydactyly in the guinea pig in developmental terms. Both assume that teratogenesis follows from differential destruction or inhibition of dominant regions, which are most susceptible to damaging influences. However, no cell destruction was observed within the organizer or reactor tissues in the cases under discussion. Obviously, the gradient theory alone is insufficient to explain the variety and the specific detail of the observed reactions. On the other hand, Harrison (99) and Lehmann (54) believe they recognize in the malformations the expression of defects in corresponding regions of the head organizers in the roof

of the archenteron. In comparing the lithium-induced defects with similar ones that are obtained through resection of parts of the dorsal lip of gastrulae, one should not overlook the fact that the lithium treatment does not produce analogous lesions in the archenteron roof. Lehmann's answer to this difficulty, his postulate of mesodermization of the presumptive notochord material, offers at best only a verbal solution. The concept of presumptive organs loses all but possibly some formal meaning, in case such organs never develop. Pasteels (67), starting from other premises, concludes that lithium depresses a general constituent factor which, by its quantitative distribution along gradients, conditions the cephalo-caudal as well as the dorso-ventral differentiation of the embryo. In an earlier paper on centrifugation of the uncleaved egg, the same investigator (66) attempted to find a more substantial expression for this factor and proposed that the gradients reflect the distribution of a substance, *organisin*, which might be identical with the sulfhydryl ribonucleoproteins. In centrifugation, this organisin should shift toward the animal pole, and therefore out of the embryo-forming area. While this might account for the general lowering of the capacity for differentiation, it offers no explanation for the selective reduction of rostral parts.

Embryologically, the defective development of the head follows incomplete gastrulation, irrespective of the nature of the employed experimental factor. The author (93) therefore concluded that the *degree of invagination* determines the extent of differentiation. A complete embryo forms if the organizer is carried with the archenteron to the rostral head level. The same organizer, invaginated only up to ear level, induces the differentiation of a microcephalus, with normal arrangements starting in the rhombencephalic region. The total absence of the notochord in acephali proves that presumptive notochord and organizer cannot be identical cell complexes.

The depth of invagination can gain its morphogenic importance only in collaboration with some sort of morphogenetic field, which obviously radiates from the animal pole. Spemann (78), who implanted dorsal blastopore lip into the ventral epiblast of newt gastrulae, found that the nearer to the animal pole the organizer was placed, the more rostral was the type of the induced organs. He recognized the influence of the host environment on the special differentiation of the induced secondary embryo; but neither his nor later experiments by Holtfreter (49) solved the question of whether the regional host influence is due to an independent "axial gradient" or whether it issued from the organization field of the primary embryo. This alternative is cleared up and decided in favor of the first interpretation by the overripeness experiments, which show that deficient gastrulation causes rostral reductions not only in accessory but also in primary, and even in single embryos. The determination levels govern uniformly the differentiation of all embryos arising on one egg.

It is now evident that there exist two forms of abnormal gastrulation. Failure of invagination to carry the organizer close to the animal pole is greatly different from incomplete epiboly, i.e., belated or permanently deficient engulfment of the yolk plug. The former leads to the defects of the head, the latter to spina bifida and caudal duplication. Both types of malformations may be observed in the same culture and sometimes co-exist in the same embryo.

Time relationships deserve special attention. Of the many agents that have been shown to produce some similar effects, such as uterine overripeness, only CO_2 treatments and possibly centrifugation may be said to be mimetic in the sense that they change the unfertilized egg deeply enough to initiate sequences of abnormalities spreading through the entire period of embryogenesis. So far overripeness remains unique in producing such long delayed reactions as polymelia and sex reversal, near the end of the embryonic and of the larval periods, respectively (90). Obviously, overripeness affects some of the very fundamental constituents of cellular structure. It contrasts in this respect with all those agents that produce immediate and strictly limited effects. As Ancel (2) points out, most chemicals become teratogenic by causing a delay in some developmental process that happens to be at a sensitive stage. In the chick the same substance may produce an open spine above the wings, or below the legs, or it may suppress the tailbud, all according to the time of administration at the 16th, the 26th, or the 48th hour. Similarly, Landauer (53) reports that insulin, injected into the yolk sac of incubating chicken eggs, produces taillessness during the second day, deformed bill during the fourth, and short legs during the fifth day. In all these cases development returns to normal as soon as the introduced chemical has become used up, diluted, or eliminated.

d. Developmental aplasia and neoplasia.—These abnormalities are relatively seldom discussed in embryologic papers which more often deal with the anatomy than the histology of abnormal differentiation. This is at least partly a consequence of the considerable difficulty met in the application of a terminology which was largely developed for the needs of medical pathology to the conditions ob-

served in abnormal amphibian development. Anidian embryos often consist entirely of a mass of blastemic cells of one single type only. What is their relationship to cancer? At the present this question can scarcely be answered; but their so obviously pathologic cells have many traits in common with typical cancer cells and seem as a whole closer to them than to normal cells.

It is generally assumed to be characteristic of malignant cells that they outgrow the normal tissues of the individual. However, in embryos, where all cells are still multiplying, this rule would not necessarily always have to apply. Briggs (15) also points out that a papilloma does not have to grow at higher than normal rate to spread over the surface of an embryo that is affected by overripeness and develops at a decreased rate. One may further contend that rapid growth is rather a clinical feature than a basic characteristic of malignant degradation, the more so as experimentally induced cancers begin to exhibit spreading growth only after long periods of latency. Thus, there remain as most outstanding distinctive traits the breakdown of organic co-ordination and the incompleteness of cellular differentiation. In embryos, the latter would have to express itself in the persistence of an embryonic appearance of the cells. This actually observed effect is designated as *aplasia;* if combined with considerable growth, it leads to the formation of *neoplasms*. The fact that these abnormalities so often lead to the death of the embryos does not necessarily constitute malignancy in the sense of human pathology.

Experimental dissociation of growth and differentiation has been accomplished by Dürken (26) and by Pasteels (66). The former by local irradiation of the dorsal blastopore lip of early gastrulae of Triturus with ultraviolet rays, the latter by centrifuging frog eggs, obtained complete gastrulae that never formed notochords or neural tissues. Similarly, in the chick, incubation at low temperature or x-radiation (3) prevents the formation of primitive streak and germ layers, resulting in unorganized cell growths that are called anidian embryos. Needham (61), investigating the inductive effects of chemicals on chick blastoderms, obtained so-called "sterol bumps," which consisted of cuboidal epithelial cells and often attained a considerable size.

In amphibians, standard carcinogens have not so far given origin to monstrosities similar to those observed in overripe cultures. Briggs (12) made an extensive study of the effects of different concentrations of a dibenzanthracene succinate (DBAS) on cleaving and gastrulating frog eggs. Retardation of development becomes noticeable at the gastrula stage. The stronger solutions cause degeneration localized in the blastopore lips, the neural folds, and the tail-bud. This picture, especially the feature of periblastoporal necrosis, is reminiscent of the complex of reactions produced by a variety of other poisons such as formaldehyde, alcohol, $MgCl_2$, KCN, and LiCl (67). It indicates a high differential susceptibility of the mechanically most active cell complexes of gastrulae and older embryos.

The polycyclic hydrocarbon derivatives estradiol, testosterone, and DBAS form a series with decreasing injury to the nuclei and increasing damage to the cytoplasm. It is therefore remarkable that both estrone and DBAS are highly potent neuroinductors, if brought locally in contact with undifferentiated ectodermal epithelium (83).

In one of our own experimental series (Strohmaier and Witschi, unpublished results) 150 frog tadpoles were treated from the time of closure of the gill sacs until metamorphosis with 250 μg/l of benzpyrene. This culture did not differ from that of the control larvae, except that it grew slightly faster and metamorphosed distinctly earlier. The experiments with carcinogens are still too few to permit far-reaching generalizations; but at their present status they do not lend support to the suggestion of Needham (61) that neoplastic effects may be due to a progressive disturbance of steroid metabolism during uterine retention of the eggs.

Overripeness under the conditions of our experiments is probably a composite reaction of at least three factors: endogenous aging, deficient O_2 supply, and CO_2 poisoning. It was Samassa (76) who discovered that fertilized frog eggs can develop up to the blastula stage in the complete absence of oxygen. This faculty of anaerobiosis, which disappears during the gastrula and neurula stages, may have evolved together with the habit of storing eggs in the uteri. Experiments on the effect of oxygen deprivation at various developmental stages in Ambystoma (22) and in Triturus (75) show a relatively high teratologic response beginning at the late blastula stage, and up to the neurula stage. Treatments before the end of gastrulation result in various degrees of micro- and acephaly. The percentage of malformations was lower in Ambystoma than in Triturus. Obviously, the urodeles, like the anurans, become more sensitive toward O_2 deficiency about the time when gastrulation begins.

The bearing of the biochemical experiments with amphibian eggs on the interpretation of metabolic processes in morphogenesis is ably discussed in the recent books by Lehmann (55) and by

Brachet (11). The present summary confines itself to the teratogenic aspects of this work.

4. Inheritance and Teratogenesis

The relative role and importance of hereditary and environmental factors in the development of monstrosities has been the subject of extended discussions, which often remind one of similar controversies in the field of sex determination. Since genes can manifest themselves only through chain reactions, it is evident that abnormalities may be due either to initial gene mutations or to secondary interferences with the normal course of reactions by environmental factors. The final products may be as similar as if one were the copy of the other. In this sense Goldschmidt (33) proposes the term *phenocopy* for nonhereditary similes of known genetic mutations. Landauer (53), investigating hereditary and nonhereditary micromelia in the chick, refers to the possibility that both might develop by way of a glycemic (or comparable) condition at a stage of specific sensitivity of the limb buds. In the first case the condition would originate from a gene mutation; in the second instance from the injection of insulin into the yolk sac of the normal embryo on the fifth day of incubation. If so, the injection would actuate a change in the main-reaction chain of such sort that from here on its course would be identical with that of the genic modification, i.e., a true phenocopy would be produced. However, so far it was not possible to find evidence of abnormal carbohydrate conditions in genetic types of micromelia (101). The comparative analysis of the developmental histories of hereditary and purely phenotypic malformations remains an open though very promising field.

Geneticists and embryologists are equally baffled by the facts of *inconstant manifestation*. It is an almost general rule that hereditary malformations do not become equally realized in all carriers of the corresponding genic constitution. Similarly, eggs or embryos submitted to the same teratogenic agents usually differ widely in their responses. In both instances one must assume that uncontrolled supporting and modifying factors are at work. Some of them may be hereditary in nature. This is suggested by the observations of Landauer (53) and of Ancel (2) that identical treatments give different incidences of malformations in various breeds of the same species (fowl). Their observations in fact prove that in seemingly normal stock there may exist definite genetic predispositions to form certain abnormalities. Genic variations of analogous type may in part be responsible for the unequal distribution of duplications among our cultures derived from overripe frog eggs.

A number of hereditary anomalies in rodents correspond closely to the malformations which in frogs arise from defective gastrulation. The various degrees of reduction of the head allied to oto- and acephaly, whose inheritance was studied by Wright (99) in the guinea pig, present a parallel to the effects of overripeness in frog embryos described in section I, 4 (p. 768). Incomplete or delayed concrescence, which in frogs follows experimentally-induced persistence of the yolk plug, occurs in mice in the form of a large number of inherited abnormalities of the axial organs (27). Most remarkable are the cases of hereditary spina bifida and duplication of the lower body region (20, 31, 32).

As already mentioned, the manifestation of genic abnormalities is often irregular and incomplete. Since normal carriers of the gene transmit the abnormality in the same way as the visibly affected ones, manifestation cannot entirely be determined by the genes but must depend partly on environmental factors. Differentiation, taking place under conditions of a phenotypic milieu, is the resultant of many genetic and external influences (95). Studies on the already mentioned bleb strain of mice (57) illustrate how by persistence and spreading of disturbed milieu conditions over an extended period, a long sequence of malformations may arise. In this case the original abnormality may be some disturbance in the circulatory system (71) or the myelencephalon (9); but later it takes the form of eye defects, syndactyly, polydactyly, club foot, and related malformations.

Very little is known about hereditary anomalies in amphibians, though Rostand (73) has recently reported hereditary polydactyly in toads. In our own laboratory a study of a case of hereditary edema and polymelia, somewhat resembling the bleb condition, is now in progress.

From all this work one can only draw the conclusion that in teratogenesis, genetic and environmental factors are always combined and that the character of any malformation, including cancer, is partly determined by heredity. But from the viewpoint of the pathologist or the embryologist a malformation, e.g., spina bifida or microcephaly, presents a problem of developmental mechanics, whether it be of the spontaneous or the experimentally induced type.

5. Transplantation and Malignancy

The partially embryonic appearance of cancer cells has led to the belief that remnants of blastemic character, having escaped differentiation at

the proper embryonic period, might at later ages become the germs of tumorous and malignant developments. No direct observations beyond the fact that cancer cells are capable of mitotic division have been reported in favor of this hypothesis, which usually is referred to as Cohnheim's theory (18). Extraregional gonia, lost along the path of their normal migration from the yolk sac of the embryo to the gonadal folds, were specifically considered as potential cancerogenic material. But again, no actual development of such cells has come under observation (94). The theory has, however, stimulated a great volume of experimental work, designed to provide an older organism with embryonic cells by means of transplantation. The ideal cell for implantation should lack all capacity for self-differentiation and in no sense be determined, but it should be responsive to all inductive stimuli. The closest approach to this ideal are the prospective ectoderm cells around the animal pole of amphibian blastulae and early gastrulae. Holtfreter (46), transplanting them to older neurulae and tail-bud embryos, observed the differentiation of neural and of mesodermal tissues. Besides a tendency to fit into the particular environment, a capacity to form excess structures also becomes manifest. Obviously, in the older embryos differentiation fields and inductive centers are still present. They may even be more powerful, and certainly they are more widespread than at the gastrula and primitive streak stage, when neural and mesodermal determination normally take effect. Recent experiments by Waechter (84) prove that the same reactions can be observed in transplants of presumptive ectoderm into adult newts and salamanders. That inductive potencies are retained in many adult tissues had already been established through *in vitro* tests by Holtfreter (49) and by Chuang (17). The idea that cancer may start from embryonic cells which escaped from determination fields and therefore remained undifferentiated does not gain support from these experiments. Moreover, Woerdeman (98) has shown that cancer tissues (Walker rat carcinoma 256) themselves serve as potent inductors in amphibian tests.

Nevertheless, Spemann (79), Waechter (84), Andres (4), and Lehmann (56) follow Belogolowy (7) in his assertion that transplantation of normal embryonic cells into older larval or adult individuals often results in the production of cancerous growths. According to Spemann, this would seem to be the almost regular result in adult hosts. He obtained in only one case, out of an unspecified but obviously large number of successful operations, a somewhat differentiated growth, with notochord and muscle cells. Otherwise, the implants consisted, at the age of 3–4 weeks, of connective tissue, sometimes including one or more "pearls" of epithelial cells. However, Waechter, repeating these experiments, showed that embryonic cells implanted in adult newts became well established, if the transplant was relatively large. Small pieces, like those used by Spemann, remained undifferentiated and disappeared, the majority even before the fourth week. Furthermore, the same investigator found that the host developed defensive reactions which tended to destroy the implants. Indeed, her illustrations clearly support Rössle's (72) contention of the inflammatory nature of this defense. It was mentioned before that we found widespread inflammation in the case of the secondary transplantation into an adult frog, while the larvae carrying the primary implants had not shown similar reactions. The question of the importance of the age of the host needs still further clarification. It is obvious that the problem of cancerous development of normal embryonic cells in adult hosts is in a very doubtful state.

Most workers in this field more or less clearly adopt the notion that the cancer cell differs fundamentally and irreversibly (24) from all normal cells, including the embryonic cell. Therefore, transplantation alone is not a sufficient explanation for disorganized growth. Referring specifically to this writer's earlier work, Dürken (25) maintained that overripeness of the egg must be the decisive factor, while Lehmann (56) suggested that O_2 deficiency or temporary accumulation of CO_2 in the freshly introduced implant may bring about a change in the nature of the implanted cells. Unfortunately, these assumptions have not been specifically verified in the experiments of the two investigators and the pathologic or malignant nature of the growth resulting from the implants is in neither case clearly defined or established beyond possible doubt.

Holtfreter (50) found that the presumptive ectoderm, if cultured in physiologic salt solution, loses its capacity of responding to inductive stimuli at about the same rate as if it were left in the embryo. However, under the latter condition its cells would have become determined and differentiated, while *in vitro* they retain the undifferentiated, embryonic form. Holtfreter believes that they have approached the character of cancer cells. Earle (28), culturing subcutaneous fibroblasts of the mouse in heterologous culture fluids for periods as long as 4 years, found upon reimplantation in mice that some cells had changed from normal to malignant. This occurred especially on the addition of 20-methylcholanthrene, but in one instance

also in the control culture. Following ideas developed by Fischer (29) in his work on the occasional malignant development of transplanted normal mammary tissue, one might suspect that some cancer cell was already present in the original explant, and that the condition of culture in heterologous media selectively favored the malignant over the normal cell. Evidently, Fischer's theory only evades the question of the origin of the cancer cell and seems scarcely applicable in the case reported by Gey *et al.* (30), where in carefully controlled cultures of normal connective tissue of the rat two strains of malignant cells appeared, one after 8 months, the other after $2\frac{1}{2}$ years. If in this and in similar cases one speaks of *spontaneous* conversion, it only means that the mechanics of these mutations needs further exploration.

Like the older attempts to provide the Cohnheim theory with some experimental support, so have also the recent transplantations of parts of amphibian blastulae failed to substantiate the claim that cancer starts from normal embryonic cells which escaped from external controls, determination fields, organizers, and inductors. The negative result is in favor of the assumption that cancer starts from mutated cells with changed reaction norms. The physiologic conditions attending transplantation, such as wound healing, inflammation, and inefficient exchange of respiratory gases, may favor cancerous development, where precancerous cells or tissues become exposed. But so far no good evidence seems to have been presented in support of the view that such conditions by themselves are sufficient to transform normal into malignant cells.

III. CONCLUSIONS

It is evident that overripeness of the egg produces developmental abnormalities of widely divergent nature.

1. Twinning, asyntaxia, and polymelia—in short, all forms of duplication—are of inconstant manifestation and complexly determined; they are an expression of decreased cohesion and interrelation between cells as well as between parts of inductive and of organ-forming territories.

2. Apical reductions, the various degrees of microcephaly and acephaly, exhibit a slightly different pattern in twin and in single embryos. In both groups they are the immediate consequence of incomplete gastrulation; but in the twin parasite the severity of the defect is determined prevailingly by the smallness of the accessory inductor, while in single embryos it depends on the general condition of cellular degradation in the area of prospective organ formation. In other words, the decisive factor in the first group is an insufficiency of the inductive impulse, in the second group a reduced capacity of responding to induction.

3. Aplasia, the progressive failure of cells to differentiate, is the most constant effect produced by overripeness. It seems in various degrees composed of three forms of degradation: cancerous mutation, growth inhibition, and necrotic degeneration. The latter two require no further comment. The evaluation of the first one is difficult because of lack of a generally recognized concept about the nature and the characteristics of cancer. From the fact that cancerous conversions are induced by such dissimilar agents as radiation, chemicals, and virus infections, one may conclude that cancer is a specific response based on some fundamental property of the cell. This conception is supported by the irreversibility of the cancerous degradation and its unaltered transmission through many cell generations, even months and years after the causative agent has ceased to act (24, 52). This leads to the further assumption that cancerous conversion is closely related to genic mutation. It may consist of a series of changes in structural elements which, like chromosomal genes, are self-duplicating and pass—*sui generis*—from mother to daughter cells during the mitotic process. The physico-chemical theory of cancerogenic action by Schmidt (cf. Lacassagne [52]), in combination with the assumption that a certain number (n) of unit changes must accumulate to bring about the cancerous conversion of a cell, is possibly preparing the way to an understanding of one of the most perplexing results of the overripeness experiments. Reference is made to the fact that some eggs are still developing normally several days after others, taken from the same uterus, already were grossly overripe (cf. pp. 771–73). This would be hard to understand if the effect were a case of slow asphyxiation or poisoning, but it is exactly the type of distribution that one should expect on the basis of the bombardment principle, i.e., if a number (n) of "hits" on a limited number of submicroscopic elements is necessary to change a normal into an abnormal egg. The "hit" in this instance would have to consist in the direct exposure of one of the particular self-duplicating elements (plasmogenes, plastids) to a chemical product of the anaerobic metabolism of the retained uterine egg. Following further Schmidt's trend of thought, one might tentatively assume that some compound is formed in which electron densities of the critical level of cancerogenic hydrocarbons are attained. Such speculations would, in a sense, carry back to the earlier mentioned suggestion of Needham that the basic effect of overripeness may be a change in the

steroid metabolism. They may also lead to an interpretation of the Warburg effect (R.Q. above 1.0), which is characteristically observed in abnormal embryos derived from overripe eggs (Chart 1).

It now appears probable that the rapidly growing and metastasizing cancer is only the most obvious of a large group of degradation conversions or mutations of the cell. One may even consider the possibility that these clinically important features are secondary acquisitions, the result of exogenous or endogenous growth factors combining with the abnormal reaction type of converted cells.

IV. SUMMARY

1. The first part of this paper is a report on original experimental studies on frog eggs, relating to the effects of overripeness. Overripeness is produced by the forced retention of the unfertilized eggs in the uteri, over periods up to 10 days at room temperature. The following effects on development after delayed fertilization are described:

a) Abnormalities of cleavage, gastrulation, and neurulation, leading often to dorsal asyntaxia (spina bifida and caudal duplication).
b) Twinning and polymelia.
c) Deficient organogenesis, particularly microcephaly and acephaly.
d) Cellular pathology, incomplete or entirely lacking cellular and histologic differentiation.

2. Experiments on transplantation prove that the abnormal tissues, which usually die with the damaged embryos at an early stage, may survive and grow for months if transplanted into healthy larvae or frogs. The degree of malignancy of some such transplants must be determined in continued experiments.

3. The study of the carbohydrate metabolism confirms the discovery of Samassa of the capacity of frog eggs to survive and to develop through the entire cleavage period under anaerobic conditions. It further shows that uterine eggs store considerable amounts of CO_2. Since other experimental series reveal a sensitivity of these eggs to high concentrations of CO_2, it seems probable that this metabolite is the specific teratogenic agent of overripeness. The high R.Q. values observed in cultures from overripe eggs are an expression of general tissue damage, but support also the contention that some of the effects are of the nature of cancerous conversions.

4. The frequency of manifestation of damage by overripeness increases with the temperature and with the time of retention in the uterus. The special distribution pattern of the various grades of damage is discussed in connection with the interpretation of cancerogenic action.

5. The second part of the paper consists of a review of the literature on overripeness and a discussion of the mechanics of teratogenesis, based on the results of experimental investigations.

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Figs. 5–9 represent specimens of *Rana pipiens;* all others of *R. temporaria.*

Fig. 1 shows a normal cleaving egg; all others illustrate effects of overripeness. Special descriptions are given in the text.

Fig. 1.—Normal 8-cell stage; ×15.

Fig. 2.—8-cell stage with small animal blastomeres; ×15.

Fig. 3.—12-cell stage; ×15.

Fig. 4.—Early gastrula; ×15.

Fig. 5.—Embryo, 3 days, large yolk plug; ×11.

Fig. 6.—Embryo, 3 days, asyntaxia resulting in spina bifida; ×11.

Fig. 7.—Embryo, 4 days, asyntaxia resulting in double tailbuds; ×11.

Fig. 8.—Hatching embryo, 8 days, double tail; ×11.

Fig. 9.—Larva, 14 days, spina bifida; ×5.

Figs. 10, 10*a*. Twin A, 7 days; ×11. Ventral and dorsal views.

Figs. 11, 12.—Twin B, 20 days; Cross sections through head; ×14.5.

Fig. 13.—Twin B; Cross section through midbody; ×75.

Fig. 14.—Twin H, 7 days; ×11.

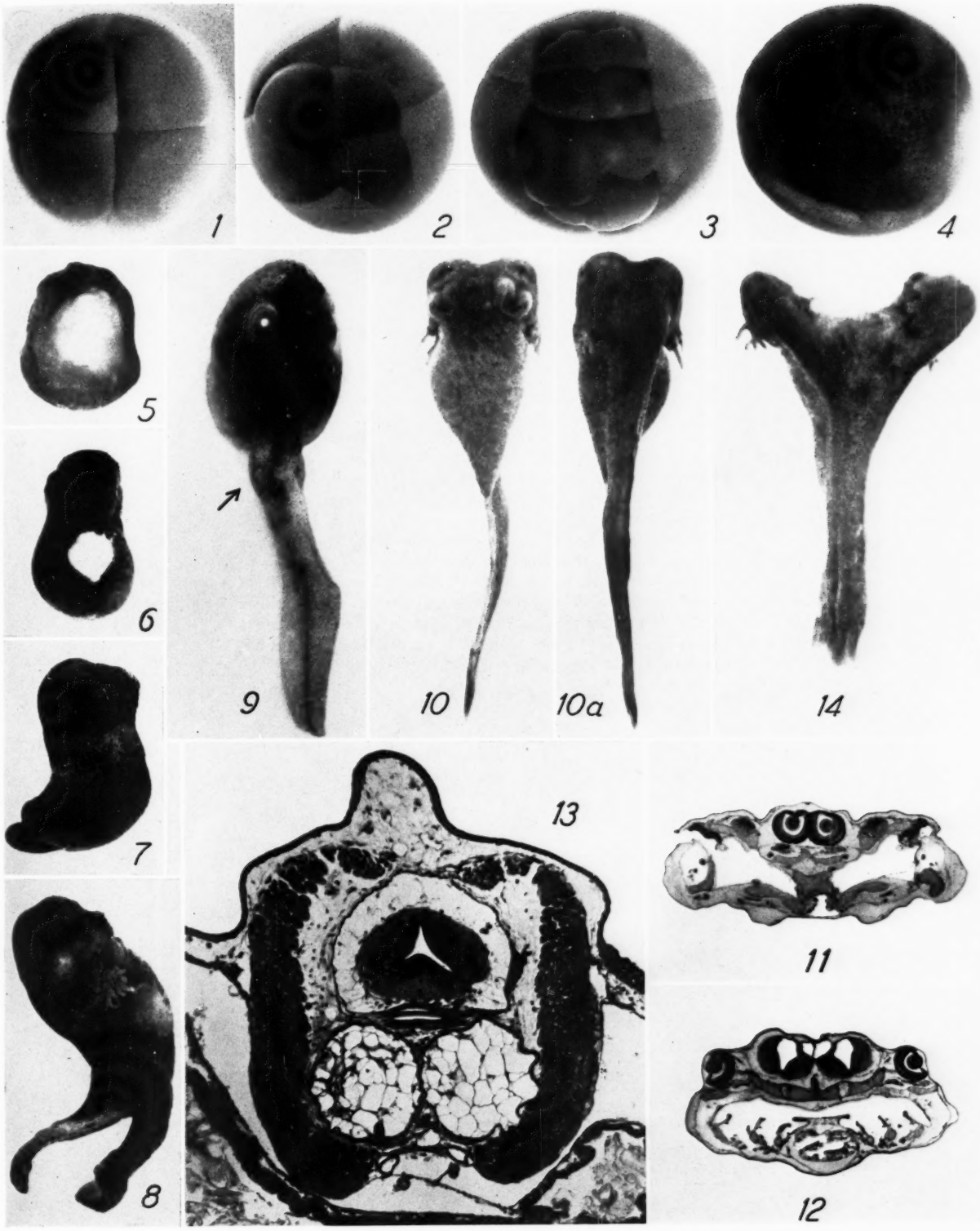
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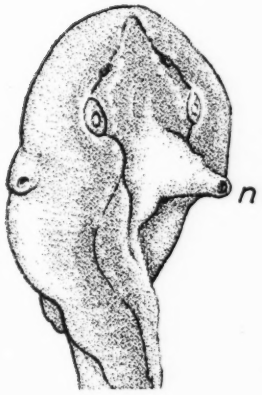
FIG. 15.—Twin Z, 21 days. Parasite on right side is anophthalmic but has a single olfactory pit.

FIG. 16.—Twin Z, ventral view. Heart with double ventricles; the single atrium (stippled) lies, partly hidden, on the dorsal side of the right ventricle; at *A* it opens separately into the left (*Vl*) and right (*Vr*) ventricles. The pharyngeal cavity is outlined in white; the left side shows the normal bronchial vestibulum, circled by four aortic arches; on the right side, the vestibulum is divided, the upper part being supplied by the third (3) the lower by the other three (4, 5, 6) of the aortic arches. The positions of the nasal rudiment (*n*) and of three otic capsules (broken lines) are indicated.

FIG. 17.—Twin P, 21 days. *A:* General arrangement of organs in a pair with caudally displaced parasite. *B;* Urogenital organs; the inner nephric ducts open into a sac without cloacal connection; some germ cells lie scattered in the triangular mesentery; parasite without organized gonads.


n

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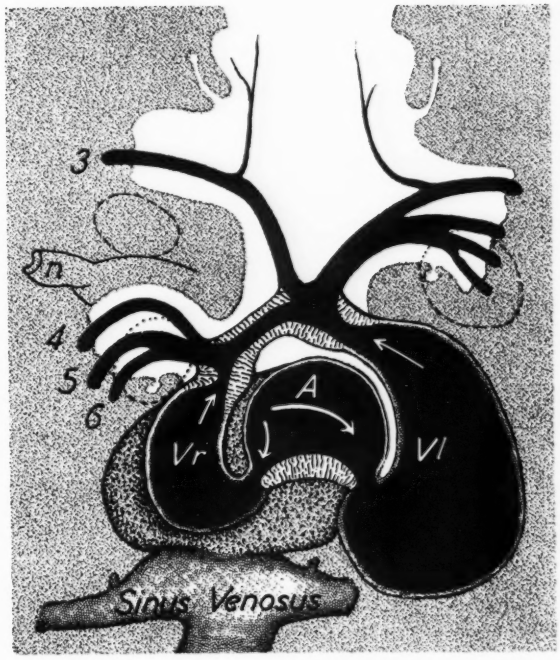
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n
4
5
6
A
Vr
Vl
Sinus Venosus

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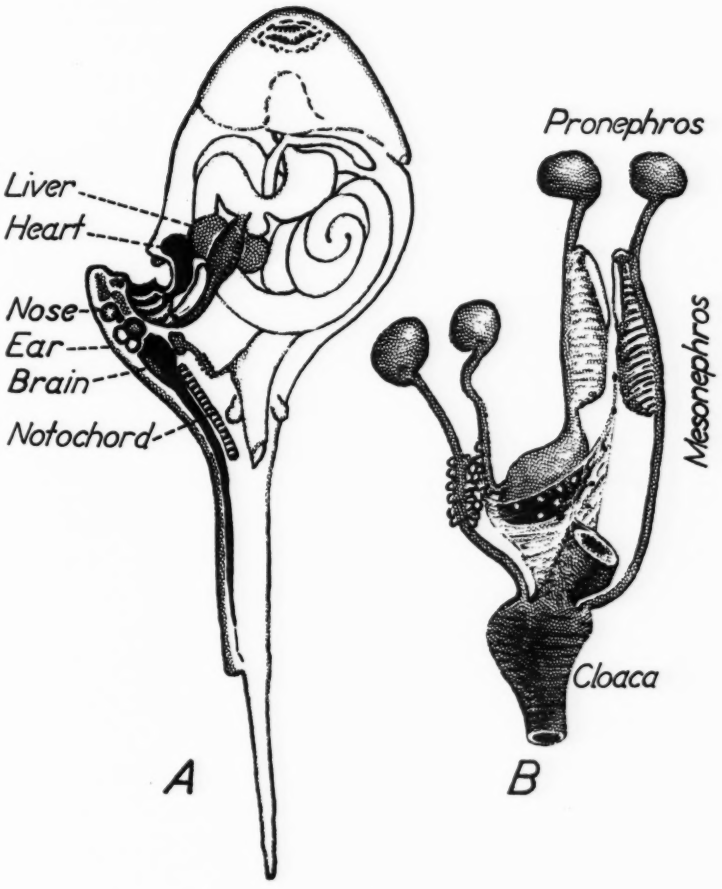
Liver
Heart
Nose
Ear
Brain
Notochord
A
Pronephros
Mesonephros
Cloaca
B

17

FIG. 18.—Twin N, 7 days; ×11.

FIG. 19.—Twin N, cross section at level of head of parasite; ×30.

FIG. 20.—Twin T, 7 days; ×11.

FIG. 21.—Larva with parasite 0, 1 month, $\times 2\frac{1}{4}$.

FIG. 22.—Twin Vb, 7 days; ×11.

FIGS. 23, 24, 25.—Twin Vg, 7 days. Parasite. Cross sections in rostral progression; ×75.

FIG. 26.—Twin Va, 7 days; near-sagittal section through parasite; ×45.

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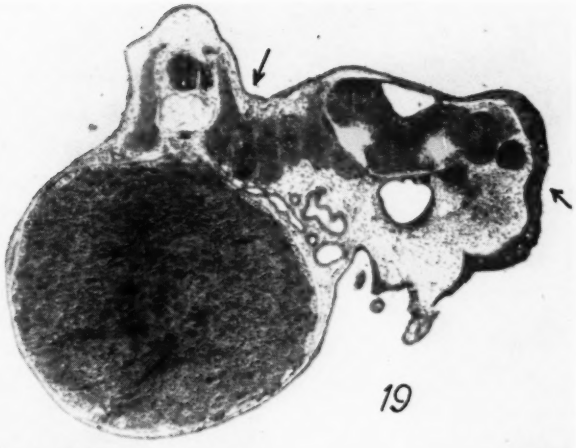

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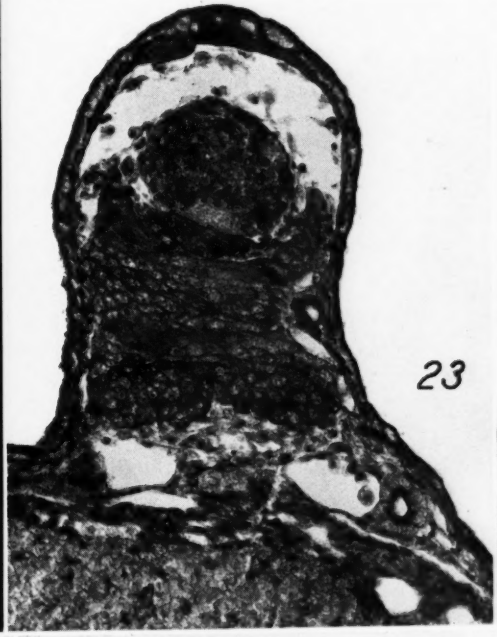

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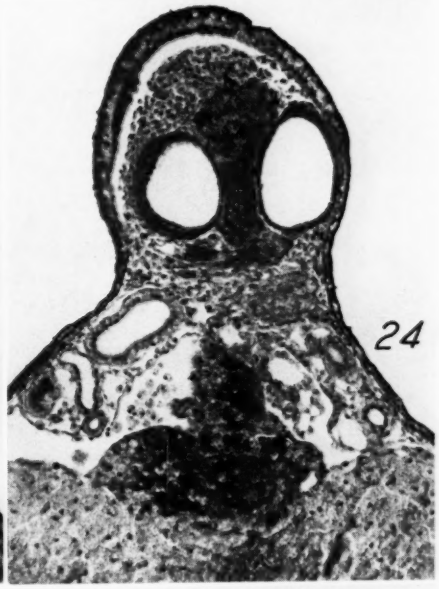

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FIG. 27.—Twin Vb, 7 days, cross section through root of tails; ×37.5.

FIG. 28.—Twin Vm, 7 days, cross section, pronephric level; ×35.

FIG. 29.—Twin Vm, part of same section, enlarged; ×150.

FIG. 30.—Twin Vm, cross section below pronephros; ×75.

FIG. 31.—Polymelia, three arms on right side, frog 3 months; ×2.

FIG. 32.—Microcephalus M54, 7 days; ×11.

FIG. 33.—Acephalus A53, 7 days; ×11.

FIG. 34.—Acephalus A27, 7 days; ×11.

FIG. 35.—Embryo E41, small hypophyseal rudiment; ×75.

FIGS. 36, 37.—Twin Vb, microphthalmia in autosite; absence of left lens; ×37.5.

FIG. 38.—Microcephalus M59, 7 days, synophthalmia, no lenses; ×75.

FIG. 39.—Microcephalus M113, larva of 2 weeks, cross section head region; ×75.

FIG. 40.—Acephalus A28, 7 days, cross sections; ×75.

FIGS. 41, 42.—Acephalus A20, 7 days; cross sections; ×75.

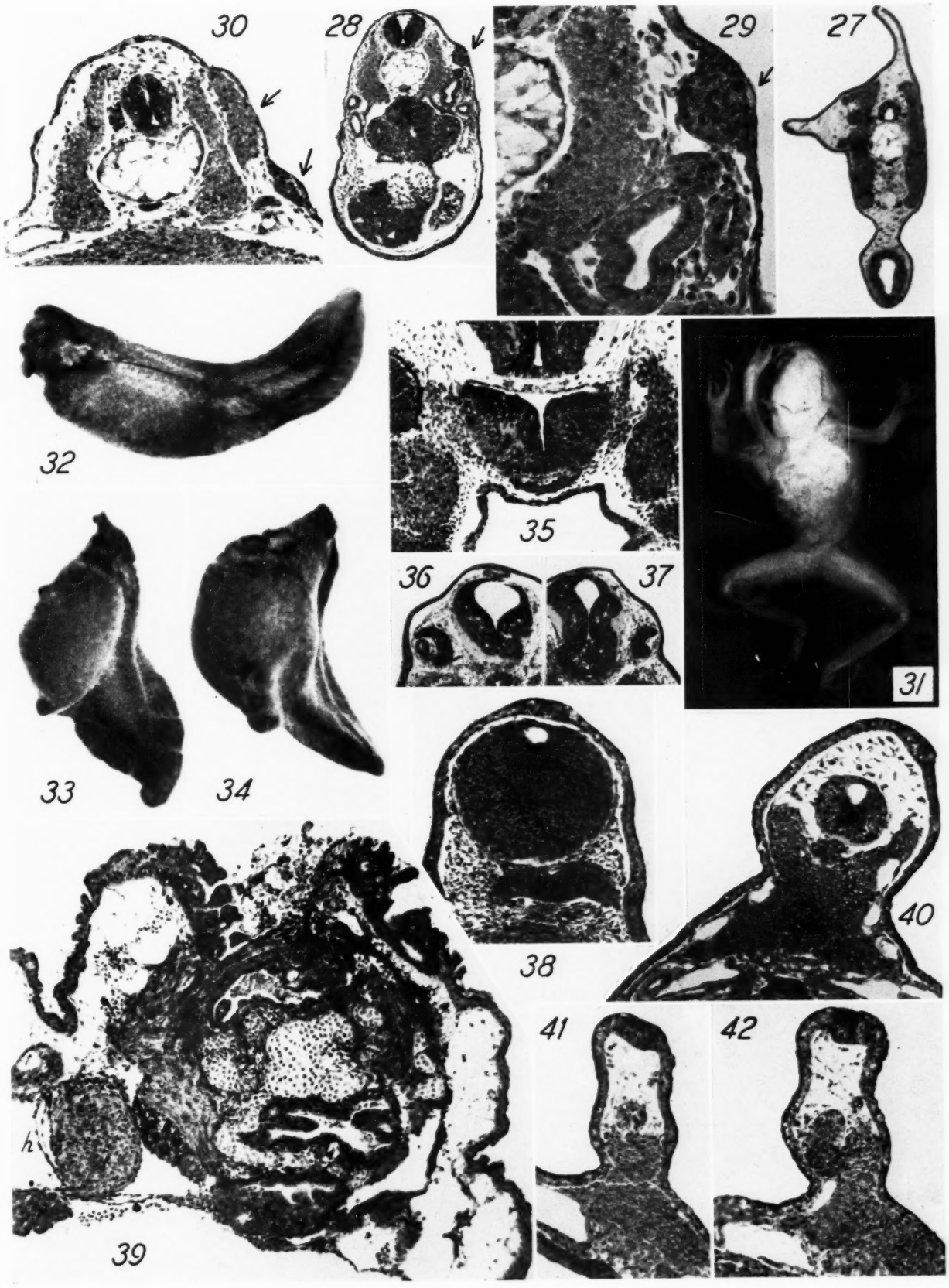
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FIG. 43.—Twin K. highly pathologic; 7 days; ×11.

FIGS. 44, 45.—Twin K, sections showing aplasia and papilloma; ×37.5.

FIG. 46.—Acephalus, A28, section through upper (pronephric) part; ×75.

FIG. 47.—Twin Vl, papilloma and placode-like thickenings; ×150.

FIGS. 48, 49.—Acephalus A28, ventral parts of cross sections; ×75.

FIGS. 50, 51.—Twin B, 20 days, papillomas on body and dorsal fin; ×187.5.

FIG. 52.—Acephalus A66, 7 days, ulceration and necrosis; ×11.

FIG. 53.—Microcephalus M113, larva 2 weeks, cross section; ×26.

FIG. 54.—Microcephalus M113, same section; ×150.

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44 h

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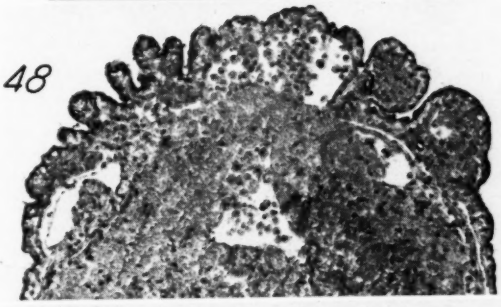

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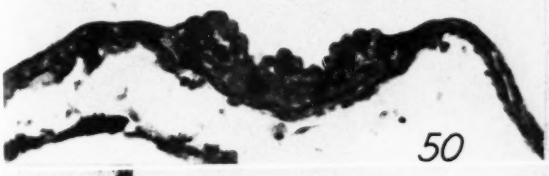

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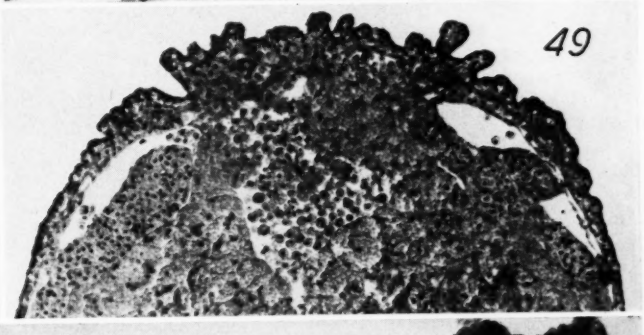

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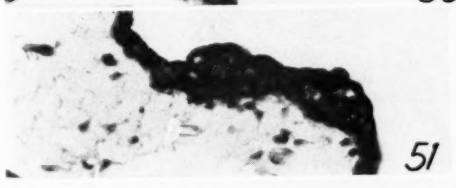

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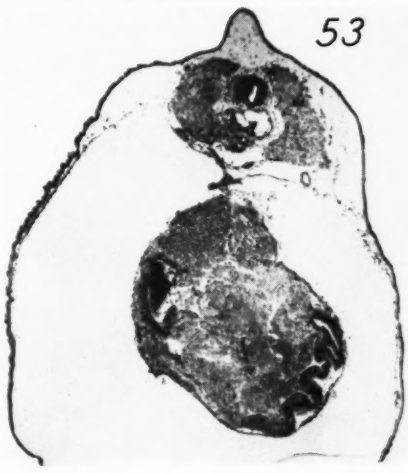

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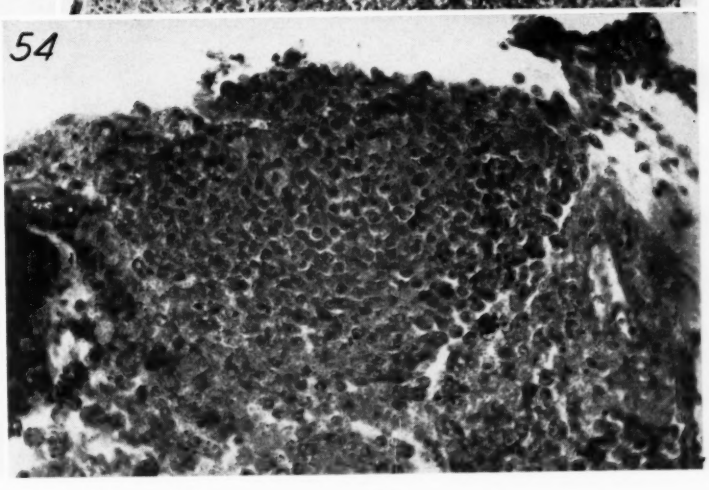

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FIG. 55.—Normal tadpole Ll with implant of overripe tissue (black) in abdominal wall; ×4.

FIG. 56.—Same tadpole Ll section with implant; ×45.

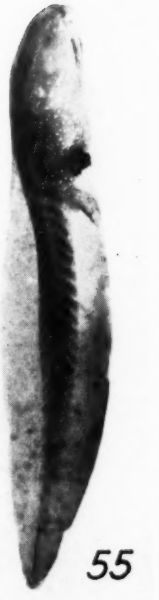
FIG. 57.—Implant with notochord in older tadpole (L5); ×75.

FIG. 58.—Secondary implant, consisting of a lipomatoid and a melanotic part, in superficial muscle layer of body wall of adult frog (I16); ×94.

FIG. 59.—Secondary implant under skin of adult frog (I16); ×75.

FIG. 60.—Invasion of melanotic implant tissue between abdominal muscles of same frog (I16); ×150.

FIG. 61.—Granuloma in intestinal wall of same frog; ×187.5.



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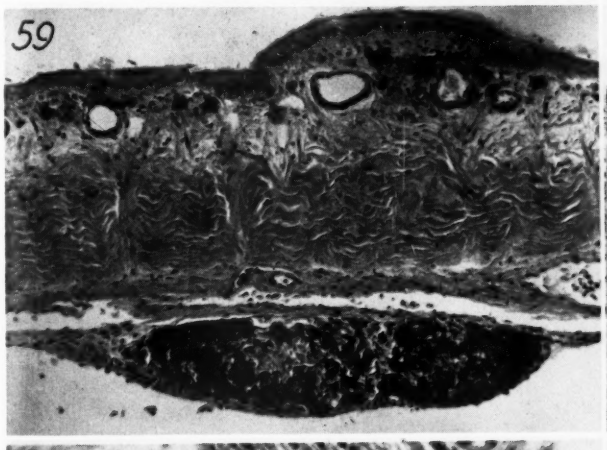

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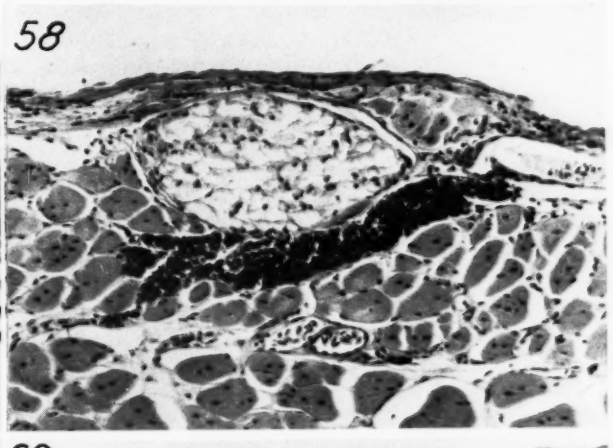

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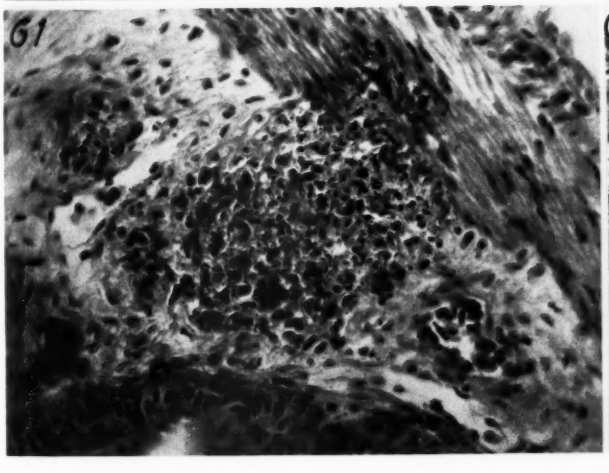

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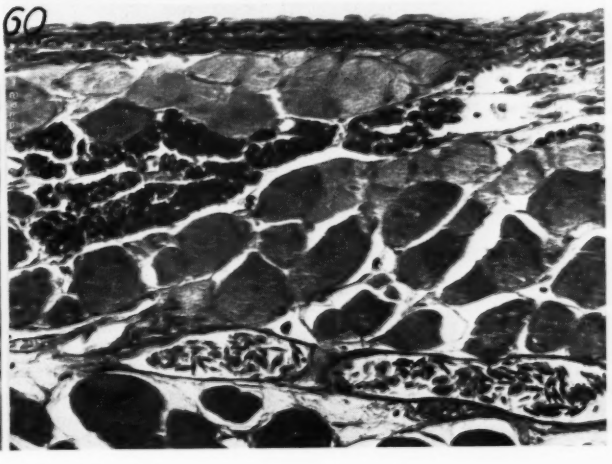

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Growth of Tumor Fragments X-radiated *in Vitro* Following Pretreatment with Cysteine

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Since the discovery of glutathione (16), many workers have demonstrated its occurrence and the significant role of sulfhydryl groups in growing tissues and organisms (13, 17, 24). More recently, the studies of Hellerman *et al.* (14), Rapkine (23), and Barron *et al.* (1, 3) have emphasized the importance of -SH groups in enzyme systems. Barron *et al.* (1) found that small amounts of x-radiation readily inhibited the action *in vitro* of enzymes whose activity was dependent upon the presence of -SH groups, and, after low doses of radiation, the enzymatic activity could be restored with sulfhydryl compounds such as glutathione. Ephrati (8) reported that cysteine and other reducing compounds added to culture media lessened the destruction *in vitro* of x-radiated bacterial toxins. Similarly, it has been shown that sylfhydryl compounds, such as thioglycollic acid and cysteine, as well as other amino acids, added to culture media will protect bacteria from the lethal action of x-rays (10, 15).

Patt *et al.* (21) and Chapman *et al.* (6) found that intravenous injection of cysteine and subcutaneous injection of glutathione, administered prior to total-body x-radiation, significantly increased survival of rats and mice. Rats bearing well implanted Walker 256 mammary carcinomas were given intravenous injections of cysteine immediately before exposure to 800 r x-radiation (25). The protection afforded the cysteine-injected rats was concomitant with the partial protection that was observed in the tumors. Protection of hair follicles in a localized area was demonstrated by Forssberg (10) following intracutaneous injection of a cysteine solution into a limited region of the dorsal skin of the guinea pig.

The experiments presented here were undertaken (11) to investigate the influence of cysteine upon the radiosensitivity of tumor fragments irradiated *in vitro* as determined by their survival and growth after implantation in nonirradiated hosts.

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MATERIALS AND METHODS

The tumor used in this study arose spontaneously in a multiparous, female, albino mouse of the Longacre-Swiss strain in 1937 at the University of Illinois (12). The tumor developed subcutaneously in the inguinal region, apparently from the mammary gland. Additional study makes it now appear more likely that the tumor is an anaplastic carcinoma, rather than an undifferentiated sarcoma. It rarely fails to "take" when inoculated into 6–10-week-old Swiss mice from the Urbana colony and generally proves fatal to the host within 3 or 4 weeks. The tumor grows invasively into smooth and striated muscle and usually gives rise to frequent metastases to the lungs and regional lymph nodes in animals that survive an actively-growing tumor for a month or more. Metastatic tumor cells have been found in the aortic lymph nodes within 5 days after transplantation of implants into the mouse uterus (12).

In the present experiments uniform, viable tumor fragments weighing 8–14 mg. were obtained aseptically from stock tumors which had been transplanted a week or 10 days before. A record of the growth of the implants was made at semi-weekly intervals from measurements of the anterior-posterior and dorsal-ventral diameters of the tumors measured to the nearest mm. with micrometer calipers. In addition, after the hosts had been sacrificed, some tumors were dissected free of surrounding tissues and weighed to the nearest milligram on a torsion balance. Since the size of an implant and the length of time that it remains out of a host are factors which can influence both its chances for survival and its early growth, care was taken to keep these factors uniform in all experiments. To increase uniformity, each series of experimental and control implants was prepared from the actively growing region of stock tumors of the same age and origin. Fragments of adequate size were thoroughly mixed and segregated into lots of 22–25, then transferred into Columbia embryological dishes in which they were irradiated.

The tumor fragments were covered with 1–2 cc. of the solution with which they were pretreated for a minimum of 20 minutes (15 minutes in the "J" series) before irradiation. Except as otherwise indicated, the implants were maintained in sterile 0.85 per cent NaCl solution. The 0.008 M cysteine solutions were made up in 0.85 per cent NaCl immediately before they were used and neutralized to pH 7 ± 0.5 with 3 N NaOH. About ⅓ of the implants not treated with cysteine were kept in cystine solutions, made by saturating 0.85 per cent NaCl with cystine. Since cystine is relatively insoluble in water, the maximum concentration of cystine in these solutions was not over 0.00046 M, and the cystine treatment did not appreciably influence survival of either irradiated or nonirradiated tumor fragments.

The radiation factors were 200 kv., 15 ma., with a 2-mm. Al filter, and a target distance of 12.7 cm. The dose rate as measured in air with a Victoreen r meter was close to 1,030 r/min.

In some experiments measured dose rates as much as 2 per cent higher or lower were employed. The tumor fragments were x-radiated while they lay in a single layer on the bottom of a culture dish, slightly covered with about 0.25 cc. of the solution with which they had been treated. Subcutaneous, bilateral axillary implants were made soon after irradiation, although occasionally fragments were kept in an icebox up to 1½ hours before implantation. Usually, the cysteine-treated tumors were the last to be implanted, but control implants clearly indicated that the time involved in making the inoculations did not influence the results appreciably. Over 400 NaCl- and cystine-treated and 100 cysteine-treated, nonirradiated implants were used as controls. In no case did a nonirradiated control implant fail to grow, nor did any host survive the effects of the control tumors.

RESULTS

The survival and growth of the tumor fragments of the "J" series, after a 15-minute treatment with 0.008 M cysteine or saline solution, and with and without radiation treatment, are summarized in Chart 1. After exposure to 4,000 r, 55 per cent of the cysteine-treated implants survived, but none of the similarly irradiated, untreated implants grew. After exposure to 3,000 r, only 70 per cent of the untreated implants grew, in contrast to 100 per cent of the cysteine-treated ones. All the untreated and cysteine-treated nonirradiated implants survived. This was also true of those irradiated with 1,000 r, but, after 2,000 r x-radiation, one implant out of twenty of both the cysteine- and untreated implants failed to grow.

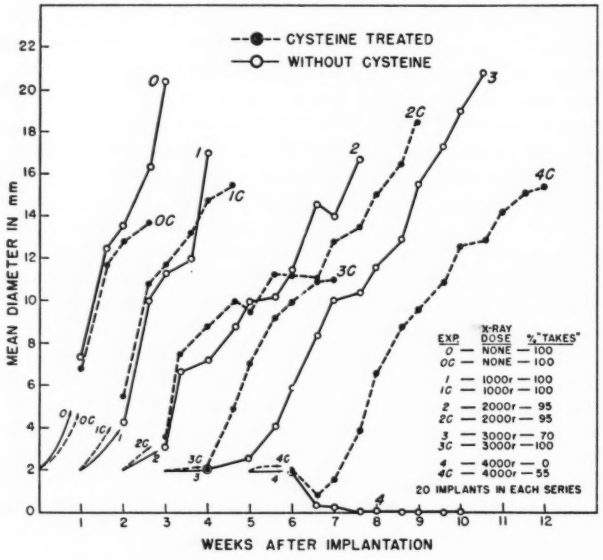
CYSTEINE TREATED
WITHOUT CYSTEINE
MEAN DIAMETER IN mm
WEEKS AFTER IMPLANTATION
EXP — X-RAY DOSE — %"TAKES"
0 — NONE — 100
0C — NONE — 100
1 — 1000r — 100
1C — 1000r — 100
2 — 2000r — 95
2C — 2000r — 95
3 — 3000r — 70
3C — 3000r — 100
4 — 4000r — 0
4C — 4000r — 55
20 IMPLANTS IN EACH SERIES

CHART 1.—Effect of x-radiation upon the growth of mouse tumor implants (J series) with and without pretreatment with 0.008 M cysteine.

Since the maximum number of growing tumors was present in the first weeks following implantation, the growth curves during this period have greater significance than those for the later period. When the ratio of tumor mass to that of the host becomes large, the host sickens, the growth of a tumor may be retarded, and is eventually terminated with the death of the host. This results in large fluctuations of the growth curves, especially prominent after the tumors reach an average diameter of approximately 10 mm.

It is apparent from the data of Chart 1 that, in addition to its obvious lethal action on tumor fragments, radiation notably increased the time required for an implant to initiate growth, although after growth was initiated the irradiated and nonirradiated tumor fragments grew at similar rates.

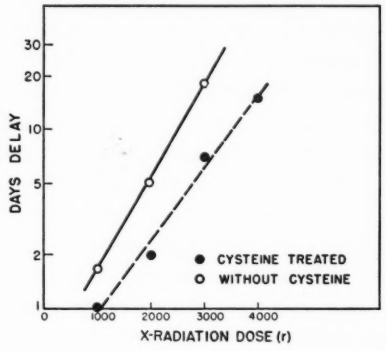

CHART 2.—Effect of x-radiation upon delay in initiation of growth of tumor implants with and without pretreatment with cysteine (semilogarithmic graph).

The larger doses of radiation increased significantly the time required for an implant to resume growth in a new host. When this delay, or "latent period," was measured as the additional time required for irradiated implants to reach the average diameter of the nonirradiated control implants at one week, it was found to be closely proportional to an exponential function of the radiation dose as shown in Chart 2. It is noteworthy that cysteine pretreatment significantly reduced by the effectiveness of each radiation dose as determined by the duration of the latent period.

Inhibition and delay of cell division are known to follow exposure of cells to moderate doses of radiation and must account to some extent for the increase in time required for an irradiated implant to resume growth. It is also known that implant size is an important factor in determining the apparent latent period; usually the smallest implants show the longest "latency." For example, a slice of tumor was thoroughly triturated in saline solution, and 0.1 ml. of the brei containing an estimated 24,000 mostly isolated cells was inoculated subcutaneously into young mice. Only a few of these implants grew, and the appearance of detectable growths was delayed 7–10 days beyond

that of the 8–12-mg. control implants. From the data of Chart 1, it is evident that the lethal action of the radiation and, by inference, a concomitant effective reduction of implant size, as measured by the number of potentially viable cells, increased with dosage. Thus, the observed extension of the latent period by irradiation of tumor implants can be related to a temporary retardation of cellular processes associated with cell division and growth and to the death of sufficient cells to cause an effective reduction in implant size.

The data of all the experiments on survival of tumor fragments after irradiation with and without cysteine are summarized in the curves plotted in Chart 3. The curves[1] were fitted from points calculated from probit transformations of the data. The mean survival percentages for each radiation dose fall very close to the calculated curves. At radiation doses of 2,000 r or less, treatment with cysteine had little effect upon the number of "takes." At doses above 2,000 r, protection against the effects of x-radiation was clearly demonstrated by the significantly greater survival of the cysteine-treated implants as compared to those not treated with cysteine. Of 160 noncysteine-treated implants, none grew after irradiation with 4,000 r, although 32 of 140, or 23 per cent, of the cysteine-treated implants survived the effects of the same amount of radiation. After 3,000 r, 39 per cent and after 3,500 r, 38 per cent more of the cysteine-treated implants survived than did their respective control implants.

Analysis of the tumor-survival curves yields the following statistics: LD_{50} for controls 2,975 $\pm$ 49 r; with cysteine, 3,544 $\pm$ 55 r. The difference is 569 r, or about 8 times its standard deviation of $\pm$74 r, and therefore highly significant. Analysis by Tippett's method (26) yielded the information that the apparent difference in the slope of the two curves is not significant ($0.3 > P > 0.2$). It appears then that cysteine treatment of the tumor fragments has an effect on the radiation response which is equivalent to a reduction of the dose by about 18 per cent.

Protection of tumor fragments against radiation injury by cysteine is also clearly demonstrated by the average diameters and weights of the tumors developed from the implants. Examination of the growth curves of Chart 1 reveals that, after x-radiation with 2,000 and 3,000 r, the difference in the mean diameters of the tumors developing from cysteine-treated and control implants at 2 weeks was 1.4 and 5.4 mm. These differences when analyzed by the method given by Fisher (9) were found to have a statistical significance (P values) of <0.05 and <0.001, respectively. In Table 1 are listed the average weights of the tumors that developed from irradiated and nonirradiated implants 13–24 days following implantation. After 22–23 days, the cysteine-treated, x-radiated implants (3,000 r) had grown into tumors whose average weight was nearly three times that of the untreated ones irradiated with the same dose. The ratios of the

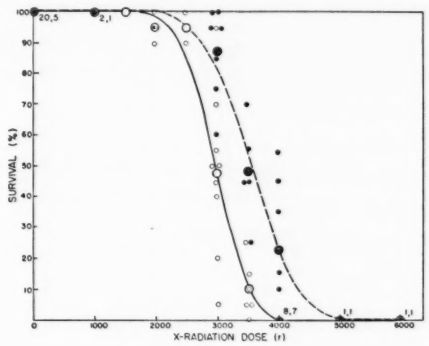

CHART 3.—Effect of x-radiation with and without cysteine pretreatment upon survival of mouse carcinoma implants. Closed circles, with cysteine; open circles, without cysteine; large circles, means; small circles, separate experiments with 20 implants; paired figures indicate number of separate experiments without cysteine, and with cysteine in respective means.

average weights of the cysteine-treated to the noncysteine-treated tumors were consistently higher in both the irradiated and nonirradiated series. The average ratio for the irradiated series was 2.91 and for the nonirradiated series, 1.33. Without cysteine pretreatment, only 44 per cent of 140 tumor fragments survived 3,000 r radiation and in 22–24 days they grew to an average weight of only 245 mg. With cysteine pretreatment, 85 per cent of 120 implants survived the same radiation and attained an average weight during the same time of 713 mg. There is, then, positive correlation between survival after exposure to radiation and tumor size. Since the slopes of the growth curves in Chart 1 are much alike, the primary factor determining differences in tumor average sizes cannot be attributed to depression of the fundamental growth rate of the tumor. However, there does appear to be a significant relationship between the effect of cysteine-pretreatment and irradiation upon the delay in initiation of growth following implantation, and this delay very significantly affects the size of tumors measured in the early stages of growth.

The larger size of the cysteine-treated, as compared to the noncysteine-treated, nonirradiated

[1] The calculations for plotting the survival curves and for their analysis were done by Miss Joan Gurian of the statistical section, Division of Biological and Medical Research, Argonne National Laboratory.

implants probably was the result of their implantation into more favorable hosts whose average age was about 3 weeks younger and whose average weight was 3 gm. less than the hosts bearing the untreated control implants. Further support for the belief that cysteine treatment alone did not influence the growth of nonirradiated implants is given in Chart 1 and by the absence of a significant difference in the average diameters of tumors that developed from an additional 60 cysteine-treated, and 100 untreated, nonirradiated implants.

Since in the experiments described above, the tumor fragments were irradiated immersed in the solution with which they were pretreated, additional experiments were undertaken to evaluate the importance of the cysteine in the ambient fluid during irradiation. Tumor fragments were prepared and placed in cystine or cysteine solutions, as described previously, for 20 minutes. Immediately before irradiation, the cysteine-treated implants were thoroughly washed for 2 minutes, by vigorous shaking, in two separate washings of 3 ml. of cysteine-free physiological salt solution. The implants were then exposed directly to 3,000 r x-radiation but with only a minimum of fluid (0.85 per cent NaCl) clinging to the tumor fragments. For comparison, two separate series with cystine- and cysteine-treated, nonwashed implants were irradiated, with the results shown in Table 2. These results demonstrate that cysteine in the solution

TABLE 1

THE INFLUENCE OF 3,000 r X-RADIATION UPON THE SURVIVAL AND GROWTH OF MOUSE TUMOR IMPLANTS WITH AND WITHOUT PRETREATMENT WITH CYSTEINE*

Without cysteine		Cysteine-treated		Statistics		
Per cent takes†	Av. wt.‡ (gm.)	Per cent takes†	Av. wt.‡ (gm.)	Ratio§	P#	P‖
55	0.216 (11)	100	0.807 (20)	3.75	<0.001	<0.001
50	0.195 (10)			4.14	<0.001	<0.001
20	0.379 (4)	75	0.944 (15)	2.43	<0.001	>0.05
5	0.388 (1)	85	0.560 (17)	1.44	<0.001	>0.05
45	0.093 (9)	95	0.702 (19)	7.55	<0.001	<0.01
95	0.449 (19)	95	0.678 (19)	1.51	1.000	>0.05
40	0.185 (8)	60	0.646 (8)	3.49	<0.05	<0.05
Summary						
44	0.245 (62)	85	0.713 (98)	2.91	<<0.001	<<0.001
Results with nonirradiated but similarly treated control implants						
100	0.876 (14)	100	1.398 (16)	1.35		<0.01
100	1.357 (7)			1.03		<0.9
100	0.929 (16)			1.12		<0.1
100	0.917 (20)	100	1.146 (14)	1.24		<0.05
Summary						
100	0.964 (57)	100	1.279 (30)	1.33		<0.001

* The statistical significance of the difference in per cent survival of the cysteine- and noncysteine-treated control tumor fragments for individual experiments and the summaries in this table and elsewhere are indicated by the "P" values which were determined graphically by the use of Binomial Probability Paper (Mosteller and Tukey [19]).

† Per cent of 20 tumor implants that grew to a diameter of 2 mm. or more in 22–24 days (irradiated implants), and in 13–14 days (nonirradiated implants).

‡ Average weight is followed by number of tumors weighed.

§ Ratio of average weight of tumors from cysteine-treated to average weight of tumors from implants not treated with cysteine.

Probability of difference in per cent "takes."

‖ Probability of difference in average weights (Fisher [9]) of tumors developed from implants which were treated with and without cysteine.

TABLE 2

THE FAILURE OF CYSTEINE IN THE AMBIENT MEDIUM TO AFFECT THE RADIOSENSITIVITY OF TUMOR FRAGMENTS EXPOSED TO 3,000 r X-RADIATION

Treatment	Irradiated in:	Exp. no.	No. of implants	Survival No.	Survival Per cent	P
20 min. in 0.85 per cent NaCl plus 0.008 M cysteine then washed in 0.85 per cent NaCl	0.85 per cent NaCl	Q_2	20	18	90	
"	"	Q_4	20	15	75	
Summary			40	33	82.5	<0.001
0.008 M cysteine	0.008 M cysteine	Q_{10}	20	13	65	
"	"	Q_{12}	20	18	90	
Summary			40	31	77.5	<0.001
Cystine (sat.)	Cystine (sat.)	Q_1	20	9	45	
"	"	Q_3	20	10	50	
Summary			40	19	47.5	

bathing the tumor fragments was not essential during the period of irradiation for protection of the implants from the effects of ionizing radiation; for the twice-washed, cysteine-treated tumor fragments survived exposure to 3,000 r in even slightly greater numbers than those irradiated in the cysteine solution (33/40 as compared to 31/40). Of the 40 implants treated with and x-radiated in cystine, only nineteen survived exposure to 3,000 r. The results of these experiments clearly indicate that cysteine in the ambient medium had no active part in protection of the tumor fragments other than to supply the cells with additional sulfhydryl compound.

These experiments lead to a brief discussion of the mechanism of the protective action of -SH compounds and of the relative effectiveness of intracellular, as opposed to extracellular cysteine or related sulfhydryl compounds, in protecting cells from ionizing radiations. The available evidence supports the view that cysteine, glutathione, BAL, and related sulfhydryl compounds readily enter cells both *in vivo* and *in vitro*. For example, Braunshtein and co-workers (4) have demonstrated the rapid entry of cysteine from the culture medium into intact tissue slices *in vitro*, by determining its synthesis into glutathione. In addition, Barron *et al.* (2) and Voegtlin *et al.* (28) have presented other evidence which can be interpreted to signify that BAL and glutathione (and presumably cysteine) readily diffuse into tissue slices *in vitro*.

The action of cysteine in protecting cells from ionizing radiations must be a chemical one, perhaps by the formation of protective combinations with essential enzymes or genes, or by the induction of other more general cellular effects (Patt *et al.* [20]). However, the evidence of the work *in vitro* with enzyme inhibitors and the well known facility of sulfhydryl compounds to react rapidly with oxidizing agents suggest that the protective action of cysteine and related sulfhydryl compounds to a considerable degree may result from the direct and immediate neutralization of peroxides and oxidizing radicals formed from ionized water molecules (Weiss [29]). It is possible that some protection may also result from the immediate and rapid reactivation of inactivated enzymes by excess -SH groups present at the time that critical intracellular enzymes are oxidized. The possibility exists also that compounds with free -SH groups may partially protect biological systems from ionizing radiations by interfering indirectly with the formation of peroxides and derived oxidizing hydroperoxyl radicals, HO_2, perhaps by reducing the concentration of dissolved oxygen available for formation of these active entities, which Burton (5) considers to be responsible for much of the effect of ionizing radiation upon biological systems.

Since the radius of diffusion of the free radicals produced by x-rays in an aqueous medium is very restricted in relation to the gross dimensions of a cell (Lea [18]) and since free radicals are very short-lived, any theory of protection by direct and immediate neutralization of oxidizing radicals requires that the compounds supplying the active -SH groups be present within the immediate region where oxidizing radicals and injurious molecular changes occur. The results of several investigations of the relative radiosensitivity of specific regions of the cell, as determined by survival and other criteria, fully support the view that the nucleus and cytoplasm are very much more sensitive and more seriously damaged by exposure to minimum effective doses of radiation than is the cell membrane (Zirkle [30], Petrová [22], and Duryee [7]). Protection of the cytoplasm and nucleus from ionizing radiations must be then of maximum importance to the viability of a cell. These considerations lead to the suggestion that chemical protection of cells from ionizing radiations afforded by cysteine and other sulfhydryl compounds is primarily the result of their intracellular action.

SUMMARY AND CONCLUSIONS

The data presented in the survival and growth of mouse tumor fragments following x-radiation *in vitro* with and without prior treatment with 0.008 M cysteine clearly demonstrate the prevention and alleviation of radiation injury of mammalian tumor cells by cysteine. The radiation dose had to be increased by about 18 per cent to effect equivalent injury of cysteine-treated implants. Delay in initiation of growth by tumor fragments following implantation was increased by radiation effects, and the increase in latency was found to be determined by an exponential function of the radiation dose. Cysteine in the ambient medium was not found to be an effective agent in reducing the radio-sensitivity of tumor fragments. The results indicate that the prevention and alleviation of the effects of ionizing radiations upon cells by pretreatment with cysteine and related compounds is due to the intracellular action of their sulfhydryl groups.

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A Comparison of the Ratios of Metaphase to Prophase in Normal and Neoplastic Tissues

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Since one of the major objectives in cancer research is to discover those characteristics of cellular morphology and physiology which distinguish the normal from the neoplastic cell, any evidence presented which indicates such differentiation is worthy of further investigation. Thus, the observation made by Timonen and Therman (14, 15) that the relative duration of the different stages of mitosis in cancer cells as expressed in the ratio of metaphases to prophases was markedly different from that of embryonic and normal proliferative tissue cells induced us (since many prepared sections of normal and malignant tissues were available) to make some observations in an effort to determine if such ratios could be relied upon as a criterion differentiating the normal from the malignant cell in division.

In a comparison of 35 cases of human normal proliferative endometrium and 174 cases of carcinoma of the female genital tract, Therman and Timonen (14) found that the ratio of metaphases to prophases (M/P) of the carcinomas was greater than the ratio in the normal endometrium. The M/P ratio for the malignant cells, which they illustrated with bar-graphs (converted into M/P ratios from their numerical data)were: 1.9, 8.2, 3.7, 1.7, and 8.1 (av., 4.7). The M/P ratios for the normal endometrium were: 1.2, 0.95, 0.73, 0.94, and 1.4 (av., 0.84). This difference they interpreted as indicating a shorter duration of the prophase stage in the neoplastic cells, and they attributed it to the more rapid formation of the division spindle.

PROCEDURE

The specimens examined were fixed in Bouin's fluid, sectioned at a thickness of 8 μ, and stained with hematoxylin. Cell counts were made with the aid of a Whipple disc at a magnification of 1,455 $\times$. All mitotic counts were made by one observer in order to minimize variations as much as possible. Only metaphases and mid- and late prophases were counted in the various specimens Sufficient nuclei were examined so that from 50 to 100 mitotic figures were counted per section. Some sections were recounted as a test for consistency, while in others, where it was possible, sections taken from various levels of the tissue were studied and counted in order to determine the variations which might be expected in a single specimen.

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EXPERIMENTAL

Tumor tissue.—Nine different tumors of the mouse, one of the rabbit, and three from human subjects were investigated.

The various tumors (Table 1) show a rather consistent degree of uniformity in the M/P ratio. Exceptions to this uniformity were found in tumors which revealed extensive areas of necrosis. Thus, No. 16, a Sarcoma 180, with a fairly large central area of necrosis, gave an M/P ratio of 6.8. A dbrB carcinoma which had been completely removed surgically 16 days post-transplant, reinserted immediately, and removed 15 days later for sectioning and staining, showed a very large center of necrosis with an exceptionally high M/P of 12.3. In another autoimplantation of a dbrB carcinoma, the periphery of viable malignant cells adjacent to the large necrotic center showed practically nothing but metaphase stages, but these showed such a large proportion of abnormalities such as stickiness, lagging, fragmentation, and overcondensation of chromosomes, it was thought inadvisable to attempt mitotic counts.

Normal tissue.—The normal proliferative tissues used for a basis of comparison were the endometrium of the monkey, the epithelial cells of the crypts of Lieberkühn of the mouse and cat, and the cells of the stratum germinativum of the esophagus of the mouse and man.

Although it appeared at the outset of these investigations that the findings of Therman and Timonen (14) were to be confirmed, it became evident as more tissues and sections were subjected to scrutiny, that the M/P ratio for normally pro-

liferating tissues was not in all preparations smaller than that found for neoplastic tissue. The M/P ratio in dividing cells of normal tissue was often equal to that of the malignant cell and was also at times considerably smaller.

An inspection of Table 2 indicates that there exists more variation in the M/P ratios for normal than for neoplastic cells.

TABLE 1

RATIOS OF METAPHASE TO PROPHASE (M/P) FOR VARIOUS TUMORS

No.	M/P	Specimen
1	3.0	Spontaneous carcinoma in DBA strain mouse
2	3.8	Recount of (1)
3	3.9	1st transplant generation of (1)
4	3.7	dbrB transplantable carcinoma in DBA strain
5	3.2	Recount of (4)
6*	12.3	dbrB autotransplant with large necrotic center
7*	6.3	dbrB autotransplant different from (6)
8	4.6	15091a carcinoma in ABC strain mouse
9	3.0	" " " " " " . Different from (8)
10	3.8	Methylcholanthrene-induced tumor in DBA strain
11	4.0	Same as (10) but induced in a different DBA individual
12	2.8	Methylcholanthrene-induced tumor in Paris strain mouse
13	4.5	Mouse Sarcoma 37
14	4.1	Sarcoma 180 in Rockland mouse
15	3.7	" " " " . Different from (14)
16*	6.8	Sarcoma 180 with necrotic center
17	4.0	Sarcoma 180 with scattered necrosis
18	3.3	Sarcoma 180 with little necrosis
19	2.4	Mouse melanoma (Harding-Passey)
20	3.1	Brown-Pearce carcinoma (metastatic nodule)
21	4.3	" " " . Different nodule from (20)
22	3.7	Adenocarcinoma of stomach (human)
23	3.7	Metastatic carcinoma of stomach to liver (human)
24	2.9	Cancer of the cervix (human)
25	3.7	Same as (24) but different section

Av. M/P=3.5 Range=2.4–4.5 S.D.=±1.7

* Not included in calculations because of necrosis.

A particularly interesting observation was made in sections No. 37, 38, and 39. In specimen No. 37, which was removed from the middle of the human esophagus, more prophases than metaphases were observed among the mitotic figures. In another section (No. 38), taken from near the cardiac sphincter, the metaphases were predominant. In comparing this to another section (No. 39) taken from the same region, it was found that the average proportion of prophases to metaphases was again encountered. With these appreciable variations found in the same tissue, it was decided to inspect malignant and normal tissues taken from the same strain of animals at different times of the day.

Comparison of M/P for dbrB carcinoma and normal esophagus in DBA mice.—The dbrB carcinomas (av. vol., 2.8 c.mm.) and esophagi were removed from DBA mice (kept in individual cages) at 2:30 A.M., 4:00 A.M., 5:00 A.M., 8:00 A.M., 11:00 A.M., 2:00 P.M., 5:00 P.M., 9:30 P.M., and 11:00 P.M. Tissue size, fixation time, sectioning and staining were kept constant for all specimens. Only nine animals were available for this experiment; however, it will be noted from an inspection of Table 3 that there is no indication of a significant difference in the M/P for normal and malignant tissue when these specimens are obtained from a highly inbred strain of animals.

TABLE 2

RATIOS OF METAPHASE TO PROPHASE (M/P) FOR NORMAL TISSUES

No.	M/P	Specimen
24	1.1	Proliferating endometrium of monkey
25	1.1	Epithelial cells in crypts of Lieberkühn (mouse)
26	1.9	" " " " " " "
27	2.0	" " " " " " "
28	3.5	" " " " " " "
29	2.8	" " " " " " "
30	3.9	" " " " " " "
31	6.0	" " " " " " "
32	3.2	" " " " " " "
33	5.5	" " " " " " "
34	5.0	" " " " " " "
35	8.0	" " " " " " (cat)
36	0.1	Esophagus (mouse)
37	0.4	Esophagus (middle) (human)
38	5.6	" (near cardiac sphincter) (human)
39	3.3	" same as (38) but different section

Av. M/P=3.5
Range =0.1–8.0
S.D. =±2.0.

TABLE 3

COMPARISON OF METAPHASE TO PROPHASE (M/P) FOR dbrB CARCINOMA AND ESOPHAGUS OF DBA MICE

No.	Time tissue removed	M/P dbrB	M/P Esophagus
1	2:30 A.M.	5.5	*
2	4:00 "	3.2	*
3	5:00 "	3.3	*
4	8:00 "	3.9	3.0
5	11:00 "	3.4	4.8
6	2:00 P.M.	3.2	4.2
7	5:00 "	3.4	3.3
8	9:30 "	2.9	*
9	11:00 "	4.1	*
Av.		3.7	3.8
Range		2.9–5.5	3.0–4.8
S.D.		±0.7	±0.7

* Number of mitoses insufficient for a reliable M/P determination.

DISCUSSION

While it appeared at first that the number of prophases in dividing neoplastic cells were fewer than those in dividing normal cells, it became evi-

dent upon continued observation that the latter can also exhibit relatively few cells in the prophase stage. Since it is generally conceded that cancer is not altogether influenced by the host's regulatory mechanism, it is perhaps to be expected that greater variations would be encountered in normal cells than in malignant cells. Although it has been found, for example, that a diurnal mitotic rhythm occurred in plants (10), in animals (1, 3, 4, 5, 13), in man (7), and in tissues cultivated *in vitro* (9), no such phenomenon has as yet been detected in malignant tissues of animals (1) and of man (8).

The variations in the relative number of prophases and metaphases in normal proliferative cells found in our histologic studies have also been observed by others. Although data given by Kaufmann *et al.* (11) showed that they observed an M/P ratio of 0.5 for both rabbit and rat corneal epithelium, Buschke *et al.* (6) found that half of the mitotic figures in the corneal epithelium of the rat consisted of metaphases. Furthermore, whereas Kaufmann observed considerable variability in the distribution of dividing cells of a single cornea and between the two corneas of the same animal, Buschke found only minor variations from cornea to cornea of the same animal.

Brues and Marble (2) observed wide variations in the percentage of mitotic figures in the liver undergoing hyperplasia, which demonstrated that single mitotic counts could not be relied upon to determine the growth rate of the organ. The M/P ratios calculated from the data of these authors from 24 hours after hepatectomy to 49 hours were: 2.3, 3.4, 2.5, 6.3, 3.5, 5.6, and 12.5.

From the available data studied concerning the relationship of prophases to metaphases, it appears that the most conspicuous feature distinguishing dividing malignant from normal cells is not so much the difference in the M/P ratios as it is the relative uniformity of these ratios for malignant cells as compared to normal cells. Although a sufficient number of determinations has not been made, this does not appear to hold for normal and malignant tissue taken from the same inbred strain of animals. This uniformity may, however, apply only to the M/P ratio, for, according to Lambert (12), the time required for the complete mitotic cycle is more variable for cancer cells than for normal cells *in vitro;* being an average of 33 minutes (range 21–50 min.) for rat fibroblasts and 56 minutes (range, 24–97 min.) for the cells of a rat sarcoma.

SUMMARY

1. The metaphase to prophase ratios of neoplastic cells showed less variation than those of normal proliferating cells.

2. That the ratio of metaphases to prophases in normal tissue cells in division is smaller than in malignant cells has not been conclusively verified by our observations.

3. There appears to be an increase in the metaphase to prophase ratio in malignant cells of tumors which contain a substantial volume of necrotic material.

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Triethylene Melamine *in Vitro* Studies

I. Mitotic Alterations Produced in Chick Fibroblast Tissue Cultures*

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As part of a cancer research program, various *in vitro* experiments have been performed with triethylene melamine in an attempt to correlate clinical and tissue culture data on this drug. This study is an adjunct to cell outgrowth measurements previously reported by Antikajian, Wright, Plummer, and Weintraub (1). The following data report specific changes produced by toxic and subtoxic concentrations of the drug on normal embryonic chick fibroblast cultures.

MATERIALS AND METHODS

Twelve-day chick heart ventricle tissue was cut into 1-mm.[3] pieces. Three fragments per coverslip were imbedded in a soft coagulum consisting of fowl plasma, human umbilical cord serum, fresh chick embryo extract, and 250 units per milliliter penicillin. Then the cultures were incubated at 37° C. on double coverslips according to the procedure described by Cameron (4). After 72 hours, those cultures with equivalent growth rates were transferred to a new series of coverslips and combined either initially, or after 48 hours, with triethylene melamine. One drop of a supernatant solution was placed on each coverslip culture. It consisted of two parts chick embryo extract, two parts human serum, and one part Tyrode solution. For drug cultures, one part Tyrode was replaced by a Tyrode solution containing triethylene melamine[1] diluted several times from a concentrated stock solution. This method did not alter the pH of 7.2, equivalent to that of the control solution. The drug was sterilized by filtering an aqueous solution through a Corning fritted glass filter. The filtrate was stored immediately at −10° C. and brought to room temperature just before use.

All cultures were fixed and stained with May-Grünwald stain after a modification of Jacobson's technic (6). Qualitative linear measurements (taken from the average of four locations per explant) of the axes of outgrowth distances were made using an ocular micrometer. Measurements were taken for outgrowing cells only and did not include the original explant. Mitotic counts were determined after a modification of the method described by Murray, de Lam, and Chargaff (8). Eight fields (two north, two south, two east, two west) of the outgrowth sheet of cells around each fragment were examined at 430× magnification, and the total number of cells and all mitotic figures and pyknotic cells counted. The mitotic index (M.I.), percentage of each mitotic stage, and percentage of pyknotic cells were determined.[2] One strain of fibroblasts, initially from the same heart strain, was used for each experimental series. Data were correlated in each table within each experiment, and involved only one strain, unless otherwise indicated.

RESULTS

Primary outgrowth toxicity studies.—First transfer cultures of fibroblasts were given initially a series of dilutions of triethylene melamine to determine effective dose ranges. The dilutions used were 0.4, 4, 10, 20, 40, 80, 100, and 200 μg/ml. The concentrations eventually used for further experimental procedures were 40, 20, and 4 μg/ml. The drug was incorporated in the supernatant solution. Outgrowth axes were measured and absolute total mitotic figures were counted for 4 days from fixed and stained cultures sacrificed at the same time every day. The results are represented in Table 1.

Preliminary experiments indicated that a solution of triethylene melamine, incubated at 37° C. for a minimum of 3 hours, produced effects equivalent to fresh drug concentrations. There is, however, a 6 per cent inactivation in solution every 24 hours at room temperature, according to data

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[1] The triethylene melamine used in this investigation was furnished through the courtesy of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.

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[2] Appreciation is extended to Dr. M. Murray and Mrs. H. Benitez, Laboratory of Cell Physiology, Columbia College of Physicians and Surgeons, New York City, for advice concerning mitotic index studies.

from Calco Chemical Division, American Cyanamid Company, New York.[3]

The lowest concentration which produced total inhibition of cell outgrowth, known as the MID, was 40 μg/ml. This term was defined by Pomerat (10) as the minimal inhibitory dose and was used as an end point for the evaluation of toxicity concentrations. Fragments treated with 40 μg/ml of triethylene melamine continued to pulsate at rates equal to those of the controls. The range of pulsation rates in control cultures, at the end of 48 hours, was from one beat per second to one beat every 20 seconds. Fragments combined with triethylene melamine pulsated at rates ranging from one beat per second to one beat every 10 seconds.

Total mitotic counts of primary outgrowth cultures revealed that there were no mitoses for the first 24 hours after exposure to 20 and 4 μg/ml of the drug, even though migration occurred (Table 1, 24 hours, *C* and *D*). Therefore, the outgrowth present at 24 hours for cultures exposed to 20 μg/ml triethylene melamine was due primarily to migration. There was complete checking of additional outgrowth, and cultures were dead at 48 hours (Table 1, 48 hours, *C*).

The least injurious dose (LID), as described by Pomerat (10), was found to be 4 μg/ml. This concentration was within the calculated therapeutic dose range which caused spleen shrinkage in a patient with chronic myelogenous leukemia (12). There was a marked increase in abnormal metaphase stages and an absence of anaphase and telophase stages in cultures treated with the LID (Table 1). The outgrowth axes of fibroblast cultures exposed to the LID are represented in Chart 1. While the initial migration, up to 24 hours, was equal for all cultures exposed initially to the drug, a decrease in migration rate was apparent after a longer period. Microscopic examination showed that there were fewer cells per unit area in the drug-treated cultures (Plate 1, Figs. 1–4). Migration was never completely checked and began anew at 72 and 96 hours after drug exposure, presumably because of the gradual inactivation of the drug at 37° C.

TABLE 1

EFFECT OF TRIETHYLENE MELAMINE ON MIGRATION AND MITOSIS OF PRIMARY OUTGROWTH CULTURES*

Exposure time	Experiment	Drug concentration (μg/ml)	Mean total outgrowth (μ)	S.D. σ (μ)	Metaphase				Anaphase	Telophase	Pyknotic cells
					Prophase	Normal	Scattered	Clumped			
24 Hours	A	Control	138	± 7.2	9	54	0	27	3	27	0
	B	40	0	± 0	0	0	0	0	0	0	0
	C	20	15	± 2.0	0	0	0	0	0	0	0
	D	4	138	± 6.4	0	0	0	0	0	0	0
48 Hours	A	Control	316	± 9.0	27	48	14	0	0	18	18
	B	40	0	± 0	0	0	0	0	0	0	0
	C	20	10	± 2.0	0	0	0	0	0	0	0
	D	4	280	± 8.7	0	9	45	18	0	0	18
72 Hours	A	Control	424	±10.0	9	90	9	10	27	45	36
	B	40	0	± 0	0	0	0	0	0	0	0
	C	20	8	± 2.5	0	0	0	0	0	0	0
	D	4	367	± 8.4	0	0	45	0	0	0	45
96 Hours	A	Control	566	± 8.8	18	81	0	9	9	0	0
	B	40	0	± 0	0	0	0	0	0	0	0
	C	20	8	± 2.6	0	0	0	0	0	0	0
	D	4	501	±10.2	0	9	0	27	0	0	206
14 Days	A	Control	1,297	± 9.6	90	198	9	9	90	180	21
	B	40	0	± 0	0	0	0	0	0	0	0
	C	20	7	± 2.4	0	0	0	0	0	0	0
	D	4	1,008	± 6.8	9	0	0	72	0	0	205

* Data for total fields of eight fragments for each experiment number, as 24A.

Mitotic counts at the LID.—The data in Table 2 indicate that there was complete inhibition of mitosis at 24 hours in cultures initially exposed to 4 μg/ml triethylene melamine (Exp. 83D). The mitotic index (M.I.) was only 0.0119 for drug-treated fibroblasts at 48 hours. The control cells at 72 hours had an M.I. of 0.0832 and the drug-treated cells an M.I. of 0.0218. The number of pyknotic cells, either early mitoses or interphase cells, increased from 2.8 per cent at 48 hours to 7 per cent in the drug series at 96 hours (Plate 2, Figs. 1 and 2). A lowered M.I., as well as direct metaphase counts, indicated that there was a metaphase arrest. All cells in division were in the early

[3] Personal communication, Clinical Research Division, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.

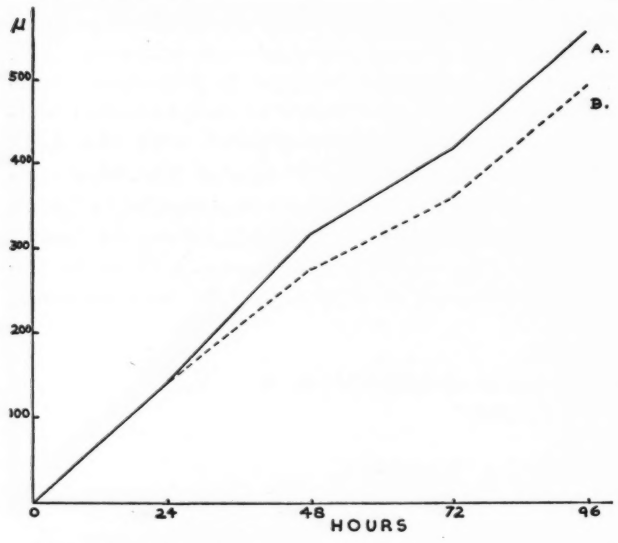

CHART 1.—Outgrowth axes of chick fibroblasts exposed to the LID of triethylene melamine. Each value represents mean outgrowth of twelve explants from two fibroblast strains, Experiment 83 and 83C.

Curve A = control cultures; Curve B = drug-exposed cultures. The standard deviations (S.D.) from control culture mean outgrowth values at 24, 48, 72, and 96 hours were ±2, ±3.5, ±5.0 and ±8.0, respectively. The S.D. from mean outgrowth values of triethylene melamine combined cultures at 24, 48, 72, and 96 hours were ±2.2, ±3.8, ±5.4, and ±7.6, respectively.

part of metaphase, with fragmented chromosomes either scattered throughout the cytoplasm or clumped in various regions in the cell (Plate 2, Figs. 3 and 4).

There was an absence of both abnormal and normal prophases, anaphases, and telophases in experimental series 83D. However, there was a significant number of multinucleated cells with small nuclei arranged in chain formation. An occasional anaphase and telophase, 12.5 per cent, was noted in the series 83P, exposed after 48 hours growth to 4 μg/ml triethylene melamine for 24 hours (Table 2, Exp. 83P).

DISCUSSION

The drug at a concentration of 4 μg/ml caused an initial inhibition of mitosis, as indicated by a mitotic index of zero. The control cultures had a mitotic index of 0.025. This effect agrees with the findings of Paterson and Boland (9), who noted a decrease in the mean number of mitoses per culture with an increase in concentration of triethylene melamine.

After 24 hours, cells recovered somewhat and attempted to divide, but disintegrated at early metaphase as indicated by the fragmentation and scattering of chromosomes and the absence of a

TABLE 2

ALTERATION IN MITOTIC VALUES IN FIBROBLAST CULTURES EXPOSED TO LEAST INHIBITING DOSE OF TRIETHYLENE MELAMINE

Exposure time (hr.)	Initial drug concentration (μg/ml)	No. cells*	No. dividing cells	Mitotic index	Metaphase†					
					Prophase†	Abnormal	Normal	Anaphase†	Telophase†	Pyknotic cells†
Experiment 83D—Initial drug exposure at time of transfer:										
24	Control	2,880	72	0.025	24 (33.3)	0	48 (66.6)	0	0	0
24	4	3,300	0	0	0	0	0	0	0	0
48	Control	3,624	96	0.026	3 (3.1)	6 (6.3)	81 (84.3)	3 (3.1)	3 (3.1)	36 (0.9)
48	4	3,708	44	0.0119‡	0	44 (100)	0	0	0	96 (2.8)
72	Control	3,315	276	0.0832	24 (8.7)	60 (21.6)	132 (47.8)	0	60 (21.6)	60 (1.8)
72	4	3,840	84	0.0218§	0	84 (100)	0	0	0	180 (4.6)
96	Control	3,192	96	0.031	3 (3.1)	0	69 (71.8)	3 (3.1)	21 (21.8)	6 (0.16)
96	4	2,928	72	0.024#	0	72 (100)	0	0	0	204 (7)
Experiment 83P—48-hour cultures exposed 24 hours to drug:*										
	Control	4,858	180	0.0374	52 (28.6)	2 (1.1)	62 (34.6)	36 (19.6)	28 (15.4)	16 (0.33)
	4	4,470	84	0.0188	2 (2.5)	64 (80)	8 (10)	6 (7.5)	4 (5)	18 (0.42)

* Total values for nine fragments per each exposure period and for each horizontal row. Total number of fragments for experiment 83D equals 72; for experiment 83P, 18 fragments.

† Figures in parentheses are per cent.

‡ 1.19 per cent of total cells in metaphase.

§ 2.2 per cent of total cells in metaphase.

2.4 Per cent of total cells in metaphase.

spindle structure. The mitotic index of 0.035 was due entirely to the presence of these disrupted metaphases.

There was no resumption of normal mitosis even after 72 hours. The mitotic index was more than 70 per cent lower than that of the controls. There was metaphase arrest, as seen by the fairly constant number of metaphase structures (2.1–2.4 per cent), even after 96 hours.

An increase in the number of pyknotic cells, with little increase in scattered metaphase structures and an absence of prophases, would appear to indicate that triethylene melamine at this concentration acts as a mitotic inhibitor. These findings resemble the results of Madsen (7) for low concentrations of nitrogen mustard in chick embryo heart fibroblast cultures. The increase in pyknotic cells with time, as well as the constant number of scattered metaphases, indicated the prolonged effect of triethylene melamine at the LID.

There were no anaphase bridge structures or normal anaphases observed for cultures initially exposed to triethylene melamine in Experiments 83B, 83C, 83K, and 83D. Telophase structures were absent, although there were significant numbers of multinucleated cells with their nuclei lined up in chain formation. This was probably caused by nuclear division without cytoplasmic division.

Failure to observe anaphase bridges in the present work, in contrast to data reported by Biesele *et al.* (2) in onion root squash preparations previously exposed to 0.1 mM/l triethylene melamine, was probably due to differences in drug concentration, length of exposure time, and the type of cell used.

The relatively slight effect of triethylene melamine on the migratory ability of fibroblasts indicated a differential sensitivity of two functions, namely, migration and mitosis, of the same cells. That mitotic arrest at interphase, for example, does not interfere with cellular growth and differentiation was shown in corneal wound healing experiments of Friedenwald, Buschke, and Scholz (5). The mechanism of cardiac muscle cell pulsations was also remarkably unaffected by MID concentrations of triethylene melamine.

Rationale for the use of triethylene melamine has been based on the presence of its three preformed ethylenimine groups. This compound, then, appears structurally related to the activated nitrogen mustards. The marked cytotoxic and tumor-inhibitory activity of triethylene melamine has been traced to the presence of these ethylenimine groups by Buckley *et al.* (3). However, the presence of the melamine nucleus in triethylene melamine alters the chemical properties from those of the nitrogen mustards, according to the report of Crossley.[4]

The mitotic changes produced by triethylene melamine may be due possibly to an interference in the nucleic acid metabolism within the interphase nucleus. Skipper, Bennett, and Wheeler (11), have found that nitrogen mustard forms insoluble reaction products with nucleic acid bases, and have isolated a guanine-nitrogen mustard reaction product after interaction of nitrogen mustard with desoxyribonucleic acid *in vitro*. Further investigation is needed to determine whether triethylene melamine reacts in this way.

SUMMARY

1. The minimal inhibitory dose (MID) of triethylene melamine in chick embryo heart fibroblast tissue cultures was 40 μg/ml. This concentration had no effect on the pulsation rate of embryo heart fragments. The least injurious dose (LID) for the same cells was 4 μg/ml.

2. Mean total outgrowth measurements revealed a slight decrease in area (about 14 per cent) for drug-treated cultures at 96 hours after initial exposure to the LID concentration of triethylene melamine.

3. The mitotic index for cultures exposed to 4 μg/ml (LID) was consistently lower than that for control cultures at 72 hours and at 96 hours. There was only a slight increase in the number of scattered metaphase cells, while there was marked increase in the number of pyknotic cells after prolonged exposure to the drug.

4. Evidence was discussed for the classification of triethylene melamine, at a concentration of 4 μg/ml, as a mitotic inhibitor, and, at higher concentrations, as a cytotoxic agent.

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Fig. 1.—72-hour control, fixed and stained, culture of chick embryo heart fibroblasts (×100).

Fig. 2.—Same culture. Note dividing cell in telophase stage (×970).

Fig. 3.—72-hour fibroblast culture of same series exposed initially to 4 μg/ml triethylene melamine (×100).

Fig. 4.—Edge of same culture showing several densely basophilic rounded cells (×970).

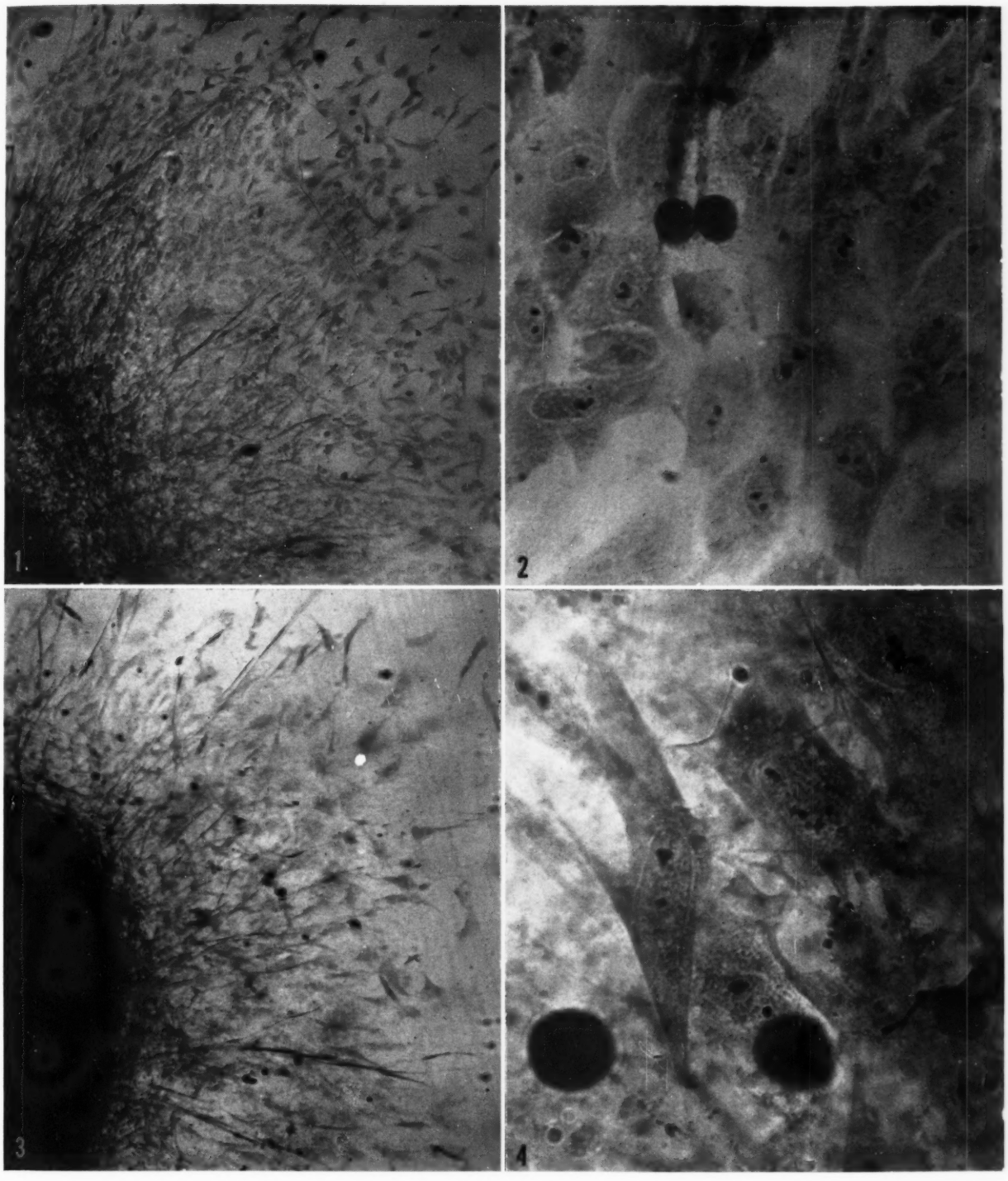
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FIG. 5.—96-hour chick embryo fibroblasts; fixed and stained, culture exposed for 48 hours to 4 μg/ml triethylene melamine ($\times$100).

FIG. 6.—6-day fixed and stained, culture exposed 96 hours to 4 μg/ml triethylene melamine ($\times$100).

FIG. 7.—Oil immersion view of stained control culture showing normal anaphase stage ($\times$970).

FIG. 8.—Oil immersion view of 96-hour culture exposed 48 hours to 4 μg/ml triethylene melamine, showing typical scattered-metaphase cell ($\times$970).

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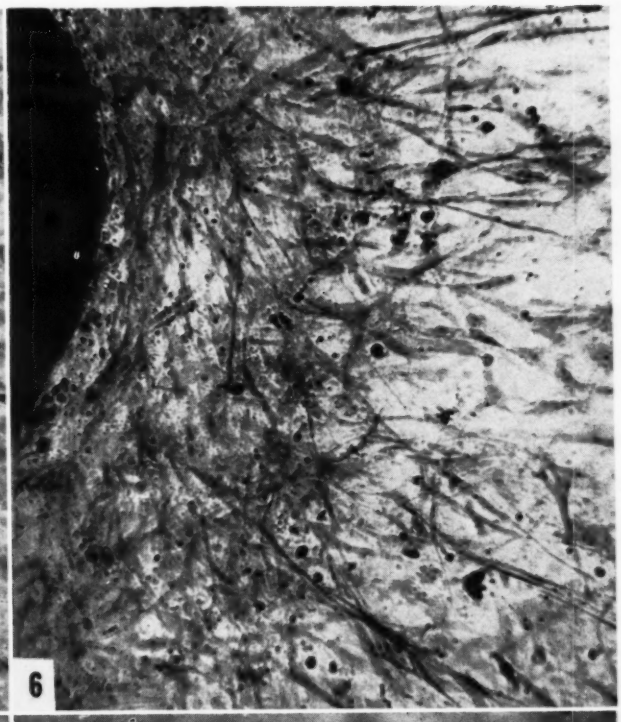
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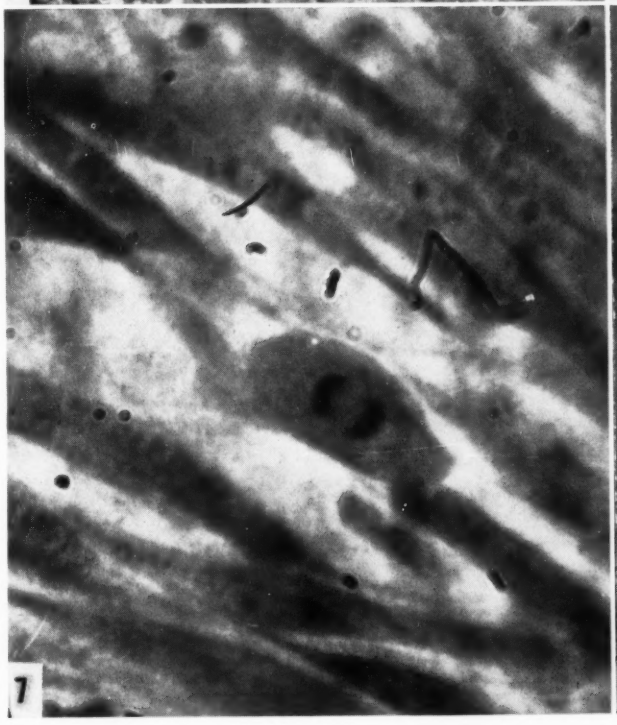
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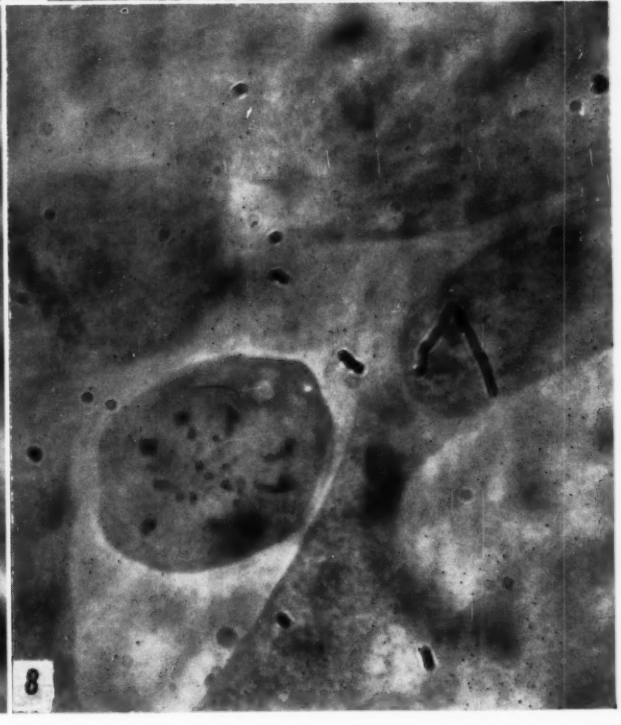
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Electrophoretic Studies on the Water-soluble Proteins of Liver during Azo Dye Carcinogenesis in the Rat*

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The concept that cancer induction involves protein abnormalities has had many proponents (13). Important evidence was the discovery by Miller and Miller (7) and Miller *et al.* (8) of the protein-bound dye occurring in the livers of rats fed azo dyes and the absence of bound dye in the hepatomas induced by this type of carcinogen. A larger portion of the dye is bound by the water-soluble proteins of liver than by any other fraction (10), indicating the importance of these proteins in the neoplastic transformation. Further indication that changes occur in the water-soluble proteins was found in the work of Griffin, Nye, Noda, and Luck (5). In a study of 3′-methyl-4-dimethylaminoazobenzene, the livers of rats, in the precancerous state, during administration of an increase in globulin and perhaps a decrease in albumin were found, as determined by $(NH_4)_2SO_4$ fractionation. Similar precancerous changes were observed for the gamma globulin and albumin of blood serum when studied electrophoretically by Cook, Griffin, and Luck (2).

Because of the role of the liver in the production of certain serum proteins, changes in the serum pattern may reflect changes in the liver; therefore, further investigation of the soluble liver proteins was undertaken. Noda (9) had found that reproducible electrophoretic diagrams could be obtained from liver extracts of the rabbit, calf, and the rat. This paper reports the extension of Noda's work to the electrophoretic study of liver extracts from normal and precancerous rats. For experiments on precancerous liver, 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) was fed for periods up to 12 weeks in an attempt to find any changes in the water-soluble proteins which might occur before tumors were induced. Experiments were also run on comparable extracts from animals fed the noncarcinogenic dye, azobenzene (AB), for 10 weeks, and on liver extracts from animals fed 2-acetylaminofluorene (2-AAF)—a different type of carcinogen—for 21 weeks. In addition to the studies on hepatic tissue, similar experiments were run on a corresponding water-soluble fraction of azo dye-induced hepatomas, blood serum from animals with hepatomas, and extracts from methylcholanthrene-induced sarcomas.

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METHODS

Adult male Holtzman-Sprague-Dawley rats were used. Either they were fed 11–12 gm/day of a purified basal diet (5), or, for periods of time up to 12 weeks, they were fed *ad libitum* the purified diet to which 0.06 per cent of 3′-methyl-4-dimethylaminoazobenzene had been added. After 12 weeks some of the animals were placed upon the purified diet and sacrificed only after tumors became palpable. Four animals were fed the basal diet to which 0.06 per cent azobenzene had been added, and three animals were fed the diet plus 0.04 per cent 2-acetylaminofluorene. Two animals (fed fox chow) were given subcutaneous injections of two $\frac{1}{2}$-cc. doses of methylcholanthrene suspended in sesame oil in a concentration of 10 mg/cc.

Preparation and analysis of extracts.—After the appropriate time interval had elapsed, the animals were fasted for 16–20 hours, anesthetized, and the livers back-perfused through the thoracic segment of the inferior vena cava with cold 0.14 M NaCl *in situ*. Blood was taken from the heart of the animals with tumors, the livers were perfused and the tumors then excised, and necrotic tissue was removed. Immediately after the animals were sacrificed, the livers were treated in essentially the manner described by Noda (9). The perfused livers or tumors from several animals (generally three to six) were pooled and homogenized with 3 times the tissue weight of ice and water in a Waring Blendor for 3–5 minutes. The temperature of the tissue suspensions or extracts was kept below 5° C. in all manipulations. After being homogenized, the tissue suspension was adjusted to pH 5 with 0.5 N acetic acid and centrifuged at 13,000 r.p.m. for 45–50 minutes in a SS-1 Servall angle centrifuge. After being centrifuged, the supernatant fluid was filtered to remove a small amount of fat, and the pH of the solution immediately adjusted to 7.4 with 0.5 N Na_2CO_3. This extract was dialyzed against 140 volumes of neutralized distilled water for 18–20 hours. The extracts were

then lyophilized, because the protein concentration was generally too low (0.2–1.0 per cent) for electrophoresis. Noda (9) found that no differences in the electrophoretic patterns of calf liver extracts occurred whether the samples were concentrated by freezing or by lyophilizing. However, considerable denaturation may occur on lyophilizing unless the samples are dialyzed quite thoroughly. Exhaustive dialysis, on the other hand, may precipitate the protein, apparently because of the acidity of ordinary distilled water. The dried proteins were stored or immediately redissolved in phosphate buffer (pH 7.7; $\Gamma/2$, 0.1) or veronal buffer (pH 8.6; $\Gamma/2$, 0.1) for electrophoresis. When the dried material was redissolved, the solution was clarified at 13,000 r.p.m. for 12 min. and a small amount of denatured protein (usually less than 3 per cent) thus removed. The protein concentration was adjusted to 1–1.5 per cent for the majority of the experiments. The extract was dialyzed against two or three changes of buffer over a period of 15–20 hours and for at least 16 hours after a final change. A total of 160 volumes of buffer was used.

The electrophoretic determinations were carried out in an 11-ml. Tiselius cell at 0.6° C. for 118–253 minutes at potential gradients from 5 to 9 v/cm. Whenever high gradients were used, the experiments were repeated at from 5 to 6 v/cm, since the ascending and descending boundaries were not symmetrical in phosphate buffer. Even at the lower gradients, however, the ascending side differed somewhat from the descending. Only the descending boundary was used for calculations of mobility and percentage composition. Conductivity and pH measurements were made on the outer buffer solution used for dialysis. The pH was measured at 25° C., the conductivity at 0.6° C.

In order to obtain the relative protein composition of each sample, the electrophoretic diagrams from liver extracts were very arbitrarily divided into four main portions. No attempt was made to construct true gaussian curves from these divisions because of the poor separation. Each of the main components was undoubtedly heterogeneous. Better separation was achieved with the tumor extracts, and the diagrams were divided into five or six components (exclusive of the epsilon boundary), depending upon the buffer used.

RESULTS

The degree of reproducibility of the electrophoretic diagrams in phosphate buffer (pH 7.7; $\Gamma/2$, 0.1) is indicated in Figure 1 (*A* to *E*) where typical patterns for the water-soluble proteins of calf and rat liver are shown.

The electrophoretic results on the water-soluble proteins of rat liver during carcinogenesis are shown in Figure 1, where the components are labeled beneath *D* and *F* and tabulated in Table 1. The electrophoretic diagrams from precancerous liver extracts resembled the control liver extract irrespective of whether 2-acetylaminofluorene was fed for 21 weeks or azo dyes up to 12 weeks. Figure 1, *F*, is an electrophoretic diagram of liver extract obtained after feeding 3′-Me-DAB to rats for 10 weeks. Small tumors were present in the animals at this time and after 12 weeks of dye feeding, yet the tissue around the tumors was similar to normal liver. The main component, No. 2, in the normal liver extracts had a mobility of -3.25 units and comprised 55 per cent of the protein.

Although no striking changes could be revealed during the precancerous period, radical differences did appear when the tissue actually became neoplastic. The electrophoretic data for liver extracts and tumor extracts are given in Table 2, and a typical diagram of hepatoma extract is shown in Figure 2, *A*. For the requisite calculations the electrophoretic diagrams of tumor extracts were divided into five segments exclusive of the epsilon boundary. In the tumor extracts the major component contained 45 per cent of the protein and had a mobility of -6.08 units. Very few liver proteins have a mobility as high as this. In fact, in the liver extracts the majority of the proteins had mobilities below -3.27 units, while in the tumor the majority of the proteins had mobilities above this figure. From the manner in which the electrophoretic diagrams from liver extracts were divided, none of the components of liver had mobilities which were the same as the proteins in the hepatoma extract, but, because of the extreme heterogeneity of the components, little can be said about the comparative mobilities of the components in the two extracts.

In attempting to characterize the proteins of the hepatomas, the resemblance of their electrophoretic diagrams to those of blood serum was noted. It occurred to us that, because of the difficulty in achieving successful perfusion of the tumors, this resemblance in the electrophoretic diagrams might result from entrapped blood. If the tumors contained blood proteins, then, in the fractionation, they would be subjected to an acidity (pH 5) which might be expected to alter the pattern somewhat. Therefore, blood serum from rats with hepatomas was diluted to approximately the concentration of the proteins present in the hepatoma extracts and the pH adjusted to 5. Some material did precipitate. This cloudy serum was then treated in exactly the same manner as the hepatoma homogenates to obtain a corresponding extract of blood serum. The results (Table 2, Fig. 2, *B*) indicated a close agreement between the mobilities of the components of serum and tumor extracts. The relative composition, however, was not the same. For simplicity, the hepatoma components were designated like serum components of corresponding mobility. This is not to imply that the components are necessarily identical, since identity cannot be assumed from the mobilities of components in complex systems. There was a larger amount of albumin in serum than of the

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EXTRACT		No. EXPERIMENTS	MOBILITIES ($-$CM.^{2} SEC.$^{-1}$ VOLT$^{-1}\times 10^{-5}$) 1	2	3	ϵ+4
Normal liver		7	6.58±0.24†	3.25±0.09	1.59±0.08	0.30±0.10
Experimental						
AB	(10 wks.)	1	6.67	3.34	1.52	0.28
2-AAF	(21 wks.)	1	6.49	3.14	1.38	0.17
3′-Me-DAB	(2 wks.)	2	6.88±0.50	3.34±0.12	1.48±0.04	0.20±0.10
	(4 wks.)	1	6.60	3.28	1.46	0.26
	(6 wks.)	4	6.66±0.12	3.27±0.30	1.57±0.35	0.30±0.27
	(10 wks.)	2	6.80±0.15	3.23±0.02	1.46±0.12	0.26±0.42
	(12 wks.)	1	6.78	3.31	1.60	0.22
			Relative protein distribution (percentage)			
Normal liver		7	3±1†	55±5	19±4	23±3
Experimental						
AB	(10 wks.)	1	4	58	17	21
2-AAF	(21 wks.)	1	2	46	24	28
3′-Me-DAB	(2 wks.)	3	3±2	51±4	20±3	26±4
	(4 wks.)	1	5	56	15	24
	(6 wks.)	4	4±2	54±3	16±1	26±2
	(10 wks.)	3	4±2	47±3	21±5	28±1
	(12 wks.)	1	4	49	20	27

* Phosphate buffer, pH 7.7; Γ/2, 0.1.

† Standard deviation calculated as $\pm wK_w$, where w equals the range and K_w is a constant from Table 1 of Dean and Dixon (3).

TABLE 2

ELECTROPHORETIC MOBILITIES AND DISTRIBUTION OF THE WATER-SOLUBLE PROTEINS OF LIVER, TUMOR, AND BLOOD SERUM FROM HEPATOMA-BEARING RATS*

EXTRACT	No. EXPERIMENTS	MOBILITIES ($-$CM.^{2} SEC.$^{-1}$ VOLT$^{-1}\times 10^{-5}$) 1	2	3	ϵ+4
Liver†	18	6.67±0.02‡	3.27±0.17	1.55±0.15	0.20±0.15
				Globulins	
		f	Albumin	α	β	γ
Hepatoma	8	7.50±0.22	6.08±0.08	3.99±0.15	2.37±0.06	1.28±0.13
Blood serum (pH 5)§	4	7.56±0.08	5.95±0.09	4.08±0.18	2.30±0.02	1.08±0.15
Blood serum+insoluble tumor proteins (pH 5)#	5	7.67±0.09	6.14±0.18	4.33±0.19	2.36±0.14	1.19±0.19
Sarcoma	2	7.44±0.51	5.69±0.34	3.83±0.20	2.44±0.08	1.38±0.04
			Relative protein distribution (percentage)			
			1	2	3	ϵ+4
Liver	20		3±1	53±4	19±3	25±3
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		f	Albumin	α	β	γ
Hepatoma	8	2±1	44±5	31±6	15±2	8±2
Blood serum (pH 5)	4	2±1	63±8	18±3	11±2	6±2
Blood serum+insoluble tumor proteins (pH 5)	5	2±1	66±9	18±7	10±2	4±2
Sarcoma	2	5±4	59±5	18±8	12±2	5±1

* Phosphate buffer, pH 7.7; Γ/2, 0.1.

† Values obtained by averaging normal and azo-dye data together.

‡ These standard deviations are equal to $\pm\sqrt{\frac{(d^2)}{n-1}}$ for $n > 10$, and $\pm wK_w$ (3) for $n < 10$.

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then lyophilized, because the protein concentration was generally too low (0.2–1.0 per cent) for electrophoresis. Noda (9) found that no differences in the electrophoretic patterns of calf liver extracts occurred whether the samples were concentrated by freezing or by lyophilizing. However, considerable denaturation may occur on lyophilizing unless the samples are dialyzed quite thoroughly. Exhaustive dialysis, on the other hand, may precipitate the protein, apparently because of the acidity of ordinary distilled water. The dried proteins were stored or immediately redissolved in phosphate buffer (pH 7.7; $\Gamma/2$, 0.1) or veronal buffer (pH 8.6; $\Gamma/2$, 0.1) for electrophoresis. When the dried material was redissolved, the solution was clarified at 13,000 r.p.m. for 12 min. and a small amount of denatured protein (usually less than 3 per cent) thus removed. The protein concentration was adjusted to 1–1.5 per cent for the majority of the experiments. The extract was dialyzed against two or three changes of buffer over a period of 15–20 hours and for at least 16 hours after a final change. A total of 160 volumes of buffer was used.

The electrophoretic determinations were carried out in an 11-ml. Tiselius cell at 0.6° C. for 118–253 minutes at potential gradients from 5 to 9 v/cm. Whenever high gradients were used, the experiments were repeated at from 5 to 6 v/cm, since the ascending and descending boundaries were not symmetrical in phosphate buffer. Even at the lower gradients, however, the ascending side differed somewhat from the descending. Only the descending boundary was used for calculations of mobility and percentage composition. Conductivity and pH measurements were made on the outer buffer solution used for dialysis. The pH was measured at 25° C., the conductivity at 0.6° C.

In order to obtain the relative protein composition of each sample, the electrophoretic diagrams from liver extracts were very arbitrarily divided into four main portions. No attempt was made to construct true gaussian curves from these divisions because of the poor separation. Each of the main components was undoubtedly heterogeneous. Better separation was achieved with the tumor extracts, and the diagrams were divided into five or six components (exclusive of the epsilon boundary), depending upon the buffer used.

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The electrophoretic results on the water-soluble proteins of rat liver during carcinogenesis are shown in Figure 1, where the components are labeled beneath *D* and *F* and tabulated in Table 1. The electrophoretic diagrams from precancerous liver extracts resembled the control liver extract irrespective of whether 2-acetylaminofluorene was fed for 21 weeks or azo dyes up to 12 weeks. Figure 1, *F*, is an electrophoretic diagram of liver extract obtained after feeding 3′-Me-DAB to rats for 10 weeks. Small tumors were present in the animals at this time and after 12 weeks of dye feeding, yet the tissue around the tumors was similar to normal liver. The main component, No. 2, in the normal liver extracts had a mobility of −3.25 units and comprised 55 per cent of the protein.

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Sarcoma	2	7.44±0.51	5.69±0.34	3.83±0.20	2.44±0.08	1.38±0.04
			Relative protein distribution (percentage)			
		1		2	3	ϵ+4
Liver	20	3±1		53±4	19±3	25±3
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Blood serum (pH 5)	4	2±1	63±8	18±3	11±2	6±2
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* Phosphate buffer, pH 7.7; $\Gamma/2$, 0.1.

† Values obtained by averaging normal and azo-dye data together.

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§ Blood serum extracted at pH 5.

Blood serum mixed with insoluble hepatoma proteins and extracted at pH 5.

component of corresponding mobility in the hepatoma extracts. On the other hand, the component with the mobility of alpha globulin was greater in relative concentration than alpha globulin of the serum.

It is possible, however, that if blood serum were present in the tumor the solubilities of its components would be influenced by the presence of the other tumor proteins in such a way as to account for the differences in relative composition that were obtained with the hepatoma extracts. In order to duplicate as closely as possible the conditions that would prevail if the blood serum proteins constituted most of the soluble proteins extractable from the tumor, blood serum was treated in the following manner. The nuclear and cytoplasmic proteins which precipitated upon extraction of the tumors at pH 5 were washed 3 times with water and centrifuged at 13,000 r.p.m. for 15 min. after each washing, to remove residual soluble proteins. The washed residue was then flash-blended and the pH adjusted to the original pH of the total tumor homogenates. Blood serum was then added and the suspension stirred for 1 hour. The quantity of serum protein mixed with the insoluble tumor protein was roughly that of the original soluble protein, and the total dilutions of the suspensions were approximately the same. After stirring, the homogenate was adjusted to pH 5 and treated exactly like the original tumor proteins. The electrophoretic diagrams of the blood serum treated in this manner did not differ from blood serum which was merely extracted at pH 5 (Table 2). We conclude that solubilization of tumor proteins by serum must have been negligible.

Because differences in the electrophoretic diagrams were found when hepatoma extracts and serum were compared in phosphate buffer, the same studies were made in veronal buffer (pH 8.6; $\Gamma/2$, 0.1) in hopes of obtaining better separations of the fractions where differences seemed to exist. The results are presented in Table 3 and Figure 2 (*D* to *F*). In veronal buffer, also, the mobilities of the components of hepatoma extract and blood serum were similar. The amount of albumin in the hepatoma extract (25 per cent) was less than that obtained in phosphate (45 per cent), because of the partial separation of the α_1-globulin from albumin. Again, the amount of albumin was less in the hepatoma extracts than in the extracted blood serum. The increase in alpha globulin in the hepatoma extract is attributable to the α_2-component and is about 3 times as great as the α_2-globulin in serum. However, there was some decrease in the α_1-globulin in the hepatoma as compared to that in the serum.

TABLE 3

ELECTROPHORETIC MOBILITIES AND DISTRIBUTION OF THE WATER-SOLUBLE PROTEINS OF TUMOR AND BLOOD SERUM FROM HEPATOMA-BEARING RATS ANALYZED IN VERONAL, pH 8.6; $\Gamma/2$, 0.1

Extract	No. experiments	Mobilities ($-$cm.^{2} sec.$^{-1}$ volt$^{-1}\times 10^{-5}$)					
				Globulins			
		f	Albumin	α	α_2	β	γ
Hepatoma	6	7.02±0.15*	6.07±0.14	5.04±0.13	3.83±0.09	2.60±0.04	1.45±0.07
Blood serum (pH 5)†	5	6.99±0.32	6.05±0.25	5.04±0.22	3.97±0.18	2.72±0.10	1.57±0.12
Blood serum+insoluble tumor proteins pH 5)‡	4	6.98±0.27	6.13±0.18	5.07±0.14	3.97±0.13	2.68±0.10	1.44±0.12
Sarcoma	1	6.68	5.74	4.34	3.20	2.52	1.38
		Relative protein distribution (percentage)					
Hepatoma	6	2±0.4*	26±4	14±2	31±6	16±0.3	11±2
Blood serum (pH 5)	5	2±0.3	45±8	22±2	10±2	14±3	7±3
Blood serum+insoluble tumor proteins (pH 5)	4	2±0.5	43±6	25±5	11±3	14±1	5±2
Sarcoma	1	2	27	44	10	11	6

* Standard deviation equals $\pm wK_w$ (3).

† Blood serum extracted at pH 5.

‡ Blood serum mixed with insoluble tumor proteins and extracted at pH 5.

In order to compare two tumor types, a similar extract of a methylcholanthrene-induced sarcoma was prepared and analyzed electrophoretically. The results are presented in Figure 2 (*C* and *F*) and Tables 2 and 3. Although the electrophoretic data are not identical for hepatoma and sarcoma proteins, in both veronal and phosphate a closer resemblance between the two tumor types is indicated than between hepatoma and liver. The sarcoma extract showed an increase in α_1-globulin over that found in blood serum, while the α_1-globulin was somewhat decreased in the hepatoma extract.

DISCUSSION

From the rather poor electrophoretic separation of those liver proteins soluble at pH 5 and the resulting subjective division of the diagrams, we

hesitate to conclude whether any alteration of components occurs during the preneoplastic period. Certainly, there are no striking differences which progress as the dye is fed and are further enhanced when tumors are actually formed. But, although no great differences between the water-soluble proteins of liver and precancerous liver were observed by electrophoretic analysis, there was a decided increase in the components having higher mobilities and a decrease in the more slowly moving proteins present in corresponding extracts of hepatomas. This conclusion is in agreement with the results of Sorof and Cohen (11) and Sorof *et al.* (12) in which they found no differences between the electrophoretic patterns of extracts from basal and preneoplastic liver, but, when tumors developed, a slowly moving component, "*h*," found in normal and in preneoplastic livers, was greatly reduced in liver tumor. In contrast to this finding, Hoffman and Schechtman (6) showed that changes did occur in the electrophoretic diagrams of preneoplastic liver. These changes were at least qualitatively in agreement with the findings of Sorof and Cohen for hepatomas, in that a faster moving component increased in amount at the expense of a more slowly moving one.

One cannot directly compare, in any detail, the results obtained by Sorof and Cohen or Hoffman and Schechtman to the results which we now report, because of the differences in extraction procedure and conditions of electrophoresis. The extracts studied by us were prepared at pH 5 and contained less protein—we sought deliberately to exclude nucleoproteins, the solubility of which is very low at pH 5. While Sorof and Cohen found at pH 7.4–7.5 that roughly 49 per cent of the total liver nitrogen was extracted as nondialyzable nitrogen, only about 16 per cent is extractable at pH 5. Noda found, in comparing liver protein extracts prepared at pH 5 to those at pH 7, that twice as much trichloroacetic acid-precipitable material was obtained at pH 7. In the hepatoma extracts we found the protein nitrogen to be only 8 per cent of the total tumor nitrogen. This would also indicate that a decrease in certain proteins has occurred in carcinogenesis. Because of the complexity of the extracts, however, it has not seemed justifiable to identify the specific components of liver which may have decreased in the hepatoma. Protein-protein interactions and protein-azo dye interactions almost certainly take place and can be presumed to result in shifts in mobility of the proteins concerned. Fractionation and identification of all the water-soluble components and rigorous determination of their mobilities would need to be accomplished first. In other words, the electrophoretic patterns obtained here are merely qualitative proof that differences exist.

The characterization of the tumor proteins is complicated also by the resemblance of the electrophoretic diagrams to those of blood serum. But whether the water-soluble proteins found in the hepatoma are the same as those in liver or are characteristic hepatoma proteins, blood serum proteins, or combinations of cytoplasmic proteins and serum proteins, one possible conclusion appears to be valid: there is a decrease of proteins extractable from the neoplastic tissue. Whether it is a partial depletion, only in the slower moving components of liver, virtually a complete disappearance leaving only blood serum, or a depletion followed by the appearance of new characteristic hepatoma proteins, it is not possible to say. That there should be a decrease is not surprising; the hepatoma is rich in nuclear material and contains a proportionally decreased amount of cytoplasm. The proteins extractable at pH 5 are perhaps exclusively those of cytoplasm and can therefore be expected to diminish.

The fact that the hepatoma extract resembled the sarcoma extract more than the liver proteins is in accord with the idea of Greenstein (4) of chemical similarity among tumors. The electrophoretic diagram of the sarcoma extract agrees well with that obtained by Barry (1) on a methylcholanthrene-induced sarcoma, although the diagrams are divided up somewhat differently. A benzpyrene-induced sarcoma also was similar, according to Barry.

SUMMARY

1. The livers of normal rats and of those in advanced 3′-methyl-4-dimethylaminoazobenzene-induced preneoplasia, were extracted at pH 5; similar extracts were prepared from hepatomas, from blood serum from hepatoma-bearing rats, and from a methylcholanthrene-induced sarcoma.

2. The electrophoretic diagrams of the extracts from normal and preneoplastic liver were similar in phosphate buffer (pH 7.7; $\Gamma/2$, 0.1).

3. The hepatoma extracts differed from the liver extracts in having less of the more slowly moving components, and more of the faster moving ones.

4. The electrophoretic diagrams of hepatoma extracts resembled those of blood serum in respect to the mobilities of the components but differed in respect to composition in both phosphate buffer (pH 7.7; $\Gamma/2$, 0.1) and veronal buffer (pH 8.6; $\Gamma/2$, 0.1). In the hepatoma extracts there was less of a component with the mobility of serum al-

bumin, and more of one with the mobility of serum α_2-globulin.

5. The electrophoretic diagrams of extracts of hepatoma and sarcoma were similar in both buffers; however, the sarcoma extract showed an increase in the component with the mobility of serum α_1-globulin, while the α_2-globulin was increased in the hepatoma extract.

ACKNOWLEDGMENT

We wish to express our thanks to Dr. Lafayette Noda, who has permitted us to draw upon the data contained in his doctoral dissertation.

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Fig. 1.—Electrophoretic diagrams of liver extracts prepared at pH 5 and analyzed in phosphate buffer, pH 7.7; $\Gamma/2$, 0.1. (Descending boundaries are on the right, ascending on the left.)

1*A*.—Calf liver extract, 5.62 v/cm, 200 min. (9).

1*B*.—Calf liver extract, 5.61 v/cm, 200 min. (9).

1*C*.—Rat liver extract, 5.38 v/cm, 220 min. (9).

1*D*.—Rat liver extract, 5.36 v/cm, 228 min.

1*E*.—Rat liver extract, 8.86 v/cm, 167 min.

1*F*.—Preneoplastic rat liver extract (3′-methyl-4-dimethylaminoazobenzene fed for 10 wks.), 5.53 v/cm, 226 min.

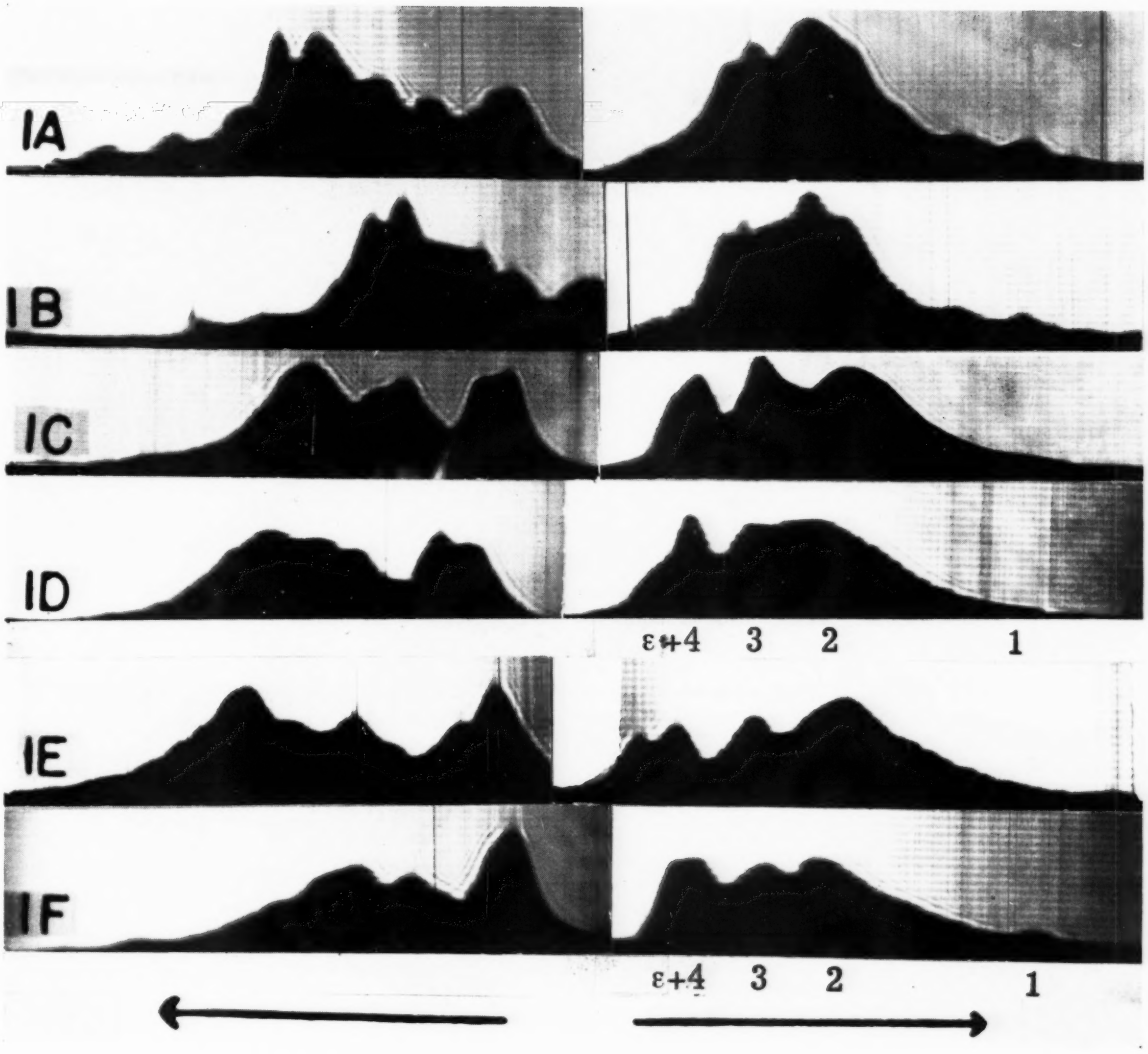
IA
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FIG. 2.—Electrophoretic diagrams of extracts from hepatomas, sarcomas, and blood serum from hepatoma-bearing rats prepared at pH 5. (Descending boundaries are on the right, ascending on the left.)

Analyzed in phosphate, pH 7.7; Γ/2, 0.1

2*A*.—Hepatoma extract, 5.27 v/cm, 224 min.

2*B*.—Blood serum extracted at pH 5, 5.35 v/cm, 226 min.

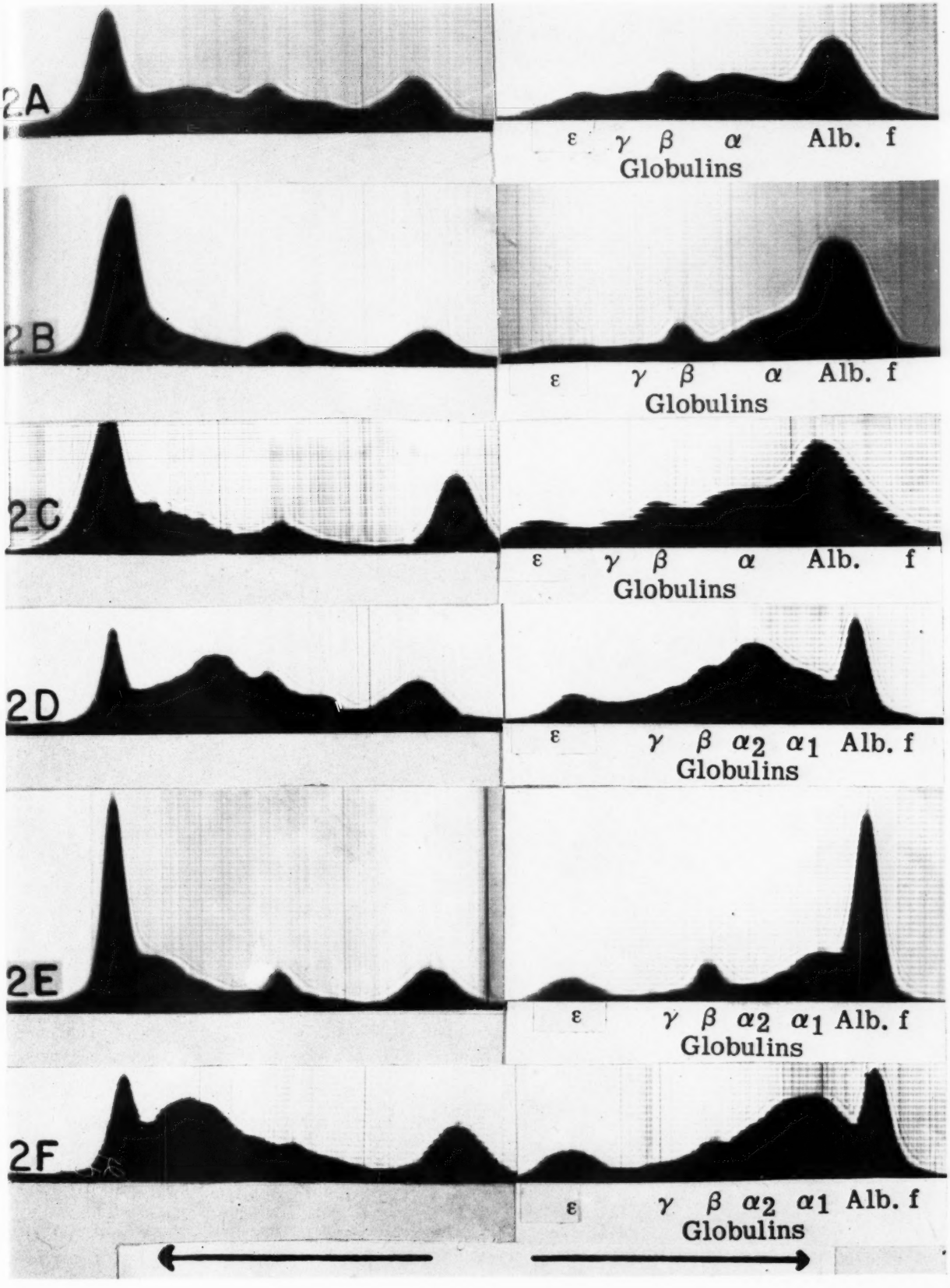
2*C*.—Sarcoma extract, 7.38 v/cm, 178 min.

Analyzed in veronal, pH 8.6; Γ/2, 0.1

2*D*.—Hepatoma extract, 5.06 v/cm, 237 min.

2*E*.—Blood serum mixed with insoluble hepatoma proteins and extracted at pH 5, 5.36 v/cm, 237 min.

2*F*.—Sarcoma extract, 4.62 v/cm, 300 min.


2A
ε γ β α Alb. f
Globulins
2B
ε γ β α Alb. f
Globulins
2C
ε γ β α Alb. f
Globulins
2D
ε γ β α2 α1 Alb. f
Globulins
2E
ε γ β α2 α1 Alb. f
Globulins
2F
ε γ β α2 α1 Alb. f
Globulins

Second Transplantations of E 0771 Mouse Carcinoma and of Brown-Pearce Rabbit Tumor*†

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When inoculations of long frozen or lyophilized tissue or fresh supernatant fluid from certain malignant tumors are injected into rabbits or mice, these hosts are rendered more susceptible to the subsequent transplant of the same tumor 1–3 weeks later (1–5, 8, 17, 23–25). This paper describes experiments designed to indicate whether a second transplantation of a fresh cellular suspension of the same tumor into a different site 1–3 weeks after a first transplant would alter the hosts' total reaction to the neoplasm. Earlier experiments by various authors were concerned with estimates of acquired immunity to a second transplant in animals negative to or showing regression of a first transplant, or in animals bearing growing tumors (13–15, 22, 26, 27). The problem of whether the second transplant affected the eventual reaction was not specifically considered in controlled experiments. The experiments of Flexner and Jobling (13, 14), Jobling (15), and Rous and Murphy (22) were sufficiently extensive and well controlled so that rearrangement of their data and analyses by statistical methods made it possible to add information on the problem. Their data will be discussed later in this paper. Our material concerns the Brown-Pearce tumor in rabbits and mammary carcinoma E 0771 in mice.

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MATERIALS AND METHODS

Two parallel series of experiments were conducted: one involved the response of 166 mice given inoculations of C57BL mouse mammary carcinoma E 0771 (Table 1); the other involved the response of 79 rabbits inoculated with the Brown-Pearce rabbit tumor (Table 2).

Mice.—In the series with the mouse mammary carcinoma, 30 mice received subcutaneous inoculations in the groin of 0.1 cc. of a 1:4 saline suspension of viable E 0771 tumor cells, and 30 mice were similarly inoculated with a saline suspension of sedimented E 0771 tumor tissue (prepared by the centrifugation at 1,200 r.p.m. for 15 minutes of a saline suspension of tumor cells, followed by the removal of the resultant supernatant fluid and the resuspension of the resultant sediment in an equal volume of saline). Approximately 8 or 15 days later, these 60 mice were given injections of viable E 0771 tumor tissue subcutaneously over the right shoulder by means of a trocar (Table 1). With E 0771 tumor tissue from the same source (i.e., 10-day-old tumor E 0771 in C57BL/6 F_1 mice from the Inbred Nucleus of the Jackson Laboratory), the 106 mice in the two control groups likewise received subcutaneous transplantions by trocar over the right shoulder by the same inoculation team. Of the 166 mice used, 60 were C57BR/cd and 106 were BALB/c (Jax subline) from the breeding rooms of the Jackson Memorial Laboratory at Bar Harbor, Maine. The 30 C57BR/cd and the 76 BALB/c controls were all the mice inoculated with C57BL subline 6 (C57BL/6) E 0771 tumor (from the Inbred Nucleus of the Jackson Laboratory) of which we have record. Mice receiving any other treatment were not included. The 60 experimental mice and 64 controls (except the last 42, marked SNCL in Table 1) were representative of some 600 mice (300 C57BR/cd and 300 BALB/c) from the same breeding rooms at the Jackson Laboratory, numbered and matched as to sex. All were 3–4 months of age when inoculated.

Rabbits.—In the Brown-Pearce tumor series,

sixteen rabbits in four experiments received, by intratesticular inoculation, 0.3 cc. of a 1:4 saline suspension of freshly excised Brown-Pearce tumor cells; and five others, Brown-Pearce tumor tissue (same site and dosage) which had been kept frozen for 18 hours at 24° F. Two weeks later the 21 experimental animals and the 22 previously uninjected controls (Table 2) were given inoculations, in the opposite testis, of 0.3 cc. of a 1:4 saline suspension of viable Brown-Pearce tumor tissue.

In four additional experiments (Table 2) nineteen rabbits received subcutaneous inoculations above the shoulder: five of 0.3 cc. of centrifugate of sedimented fresh Brown-Pearce tumor cells from which the supernatant fluid had been removed and replaced with saline, and fourteen others of a similar dosage of similarly prepared sedimented cells of Brown-Pearce tumor tissue which had been kept frozen 14 hours. Two to 3 weeks later the nineteen experimental rabbits and sixteen previously uninjected controls were given subcutaneous inoculations in the flank of 0.3 cc. of a 1:4 saline suspension of viable Brown-Pearce tumor cells.

There were originally 86 rabbits (34 chinchillas, sixteen New Zealand Whites, fourteen Brown-gray hybrids, eight Flemish, six English, four Dutch, and four miscellaneous, paired as to breed) utilized in the control and experimental series; but seven died of intercurrent disease before 21 days had elapsed following transplantation (Table 2). At post mortem examination it was possible to measure primary tumors and metastases by water displacement. The method of analysis of the rabbit tumor has been described (6).

Sections for histologic study were made of tumors used for transfer and of tumors and viscera of animals dying of tumor or having tumor at post mortem examination. After tumor transplantation the animals were examined at 5–7 days, 10–12 days, 14–15 days, 18–21 days, 28, 35, 42, 49, 56, and 63 days, when the surviving rabbits and mice were sacrificed (most of the mice without tumors at 30 days were sacrificed). Tumor growths were measured with calipers, and the product of the diameter in three dimensions in cm. was arbitrarily called the volume in cc. The error inherent in the method affected both controls and experimental animals. In the minimal lesion the product of the three dimensions was greater than 0.05 cc.

TABLE 1

SECOND TRANSPLANTATIONS OF E 0771 MAMMARY CARCINOMA (C57BL/6) INTO C57BR/CD AND BALB/C MICE 8–15 DAYS FOLLOWING THE FIRST TRANSPLANT

Experimental series									Control series						
Group	Br	TR	INT.	Mi	21	S21	60	D	Group	Br	Mi	21	S21	60	D
4101	cd	ES	15	5	1–0	0.7	0–0	1	4131	cd	5	0	0.0	0	0
4056	cd	ES	8	5	0–0	0.0	0–0	0	4481	cd	5	0	0.0	0	0
4111	cd	ES	15	5	0–2	0.4	0–0	0	4471	cd	5	0	0.0	0	0
4206	SC	ES	8	5	0–0	0.0	0–0	0	4516	cd	5	0	0.0	0	0
4236	SC	ES	15	5	0–0	0.0	0–0	0	4281	SC	5	0	0.0	0	0
4261	SC	ES	15	5	0–0	0.0	0–0	0	4431	SC	5	0	0.0	0	0
4066	cd	EE	8	5	0–0	0.0	0–0	0	4311	SC	5	0	0.0	0	0
4106	cd	EE	15	5	0–0	0.0	0–0	0	4336	SC	5	0	0.0	0	0
4116	cd	EE	15	5	1–1	3.8	0–0	1	4356	SC	5	0	0.0	0	0
4211	SC	EE	8	5	0–0	0.0	0–0	0	4326	SC	5	0	0.0	0	0
4241	SC	EE	15	5	0–0	0.0	0–0	0	4286	SC	4	0	0.0	0	0
4256	SC	EE	15	5	0–0	0.0	0–0	0	4366	SC	5	1	0.1	0	0
									4296	SC	5	3	3.0	0	2
									SNCL	cd	10	0	0.0	0	0
									SNCL	SC	32	0	0.0	0	0
Double Transplants			60	2–3	4.9	0–0	2		Controls		106	4	3.1	0	2

NOTE: Br = breed; cd = C57BR/cd mice; SC = BALB/c (Jax subline) mice; TR = type of preliminary treatment; INT. = intervals in days between 1st and 2d inoculations; ES = fresh unfrozen sedimented cells, E 0771; EE = saline emulsion of fresh unfrozen E 0771 tumor tissue; Mi = number of mice inoculated; 21 = tumor growths present 21 days after the second transplant in (*a*) the primary site in the right groin and (*b*) in the secondary site, right scapular area; S21 = summation of the volume in cc. of the tumor growths at 21 days; 60 = tumor growths in the primary and secondary sites at 60 days in surviving animals; D = deaths from tumor; mouse 4103 died 10/8/51, tumor at primary site measuring 2.85 × 2.65 × 2.35 cm.; mouse 4117 died 9/23/51, the tumor in the primary site measured 3.25 × 2.25 × 1.7 cm. and in the secondary site, 1.4 × 1.4 × 1.05 cm.; mouse 4299 died 10/29/51, the tumor measuring 3.7 × 2.4 × 1.8 cm.; mouse 4300 died 11/10/51, the tumor measuring 4.95 × 1.95 × 2.0 cm.; no metastases in the four animals.

RESULTS

The results are presented in tabular form (Tables 1 and 2). It suffices to note that no statistically significant differences in the course of the Brown-Pearce or E 0771 tumors could be ascribed to the experimental series having had a second transplantation of tumor tissue, when compared to the controls.

Mice.—The occurrence of two deaths among the 60 experimental mice, each of which had two inoculations of tumor tissue, was not inconsistent with the two deaths among the 106 controls, which had a single inoculation. The four tumor growths at

21 days, in the subscapular area of the 106 controls, were not significantly less than the three growths in the subscapular area of the 60 experimental animals ($\chi^2 = 0.1$, $N = 1$, $P = 0.75$). The average volume of the five growths in both sites in the experimental series at 21 days was 1.0 cm. and for the four growths in the controls, 0.8 cc.—not significantly different (Table 1).

Rabbits.—The primary tumor from the 41 experimental rabbits averaged 20.1 cc. at necropsy, compared to 24.7 cc. among the 38 controls (difference, 4.5 ± 15.4 cc., $t = 0.3$—not significant); the metastatic foci, 7.4, compared to 6.3 among the controls (diff., 1.1 ± 1.8, $t = 0.6$—not significant); the total tumor, 79.2 cc., compared to 52.1 cc. (diff., 27.1 ± 21.9, $t = 1.2$, $n = 77$, $P = 0.2$—not significant); the mortality, 39 per cent among the experimental and 42 per cent among the controls (not significant).

Analysis of Earlier Work with Rat and Chicken Tumors

Flexner and Jobling and Jobling (13–15) used a slowly growing transplantable rat tumor (Flexner-Jobling tumor) which caused death by progressive local growth or by metastases to distant organs in 50 per cent of the rats, usually within 60–150 days. Tumors about 1 cm. in diameter were used for transfer. These growths were between 23 and 71 days of age (average, 47),

TABLE 2

SECOND TRANSPLANTATION OF THE BROWN-PEARCE TUMOR INTRATESTICULARLY OR SUBCUTANEOUSLY IN RABBITS 12–23 DAYS FOLLOWING THE FIRST TRANSPLANT

Experimental series								Control series						
Rab.	Br	Tr-S-I	B-S	RT-LT	MF	TT	Days	Rab.	Br	B-S	PT	MF	TT	Days
1014	CH	BBRT14	BLT	34–2	21	64	33–19D	1032	CH	BLT	41	9	88	52D
1016	CH	BBRT14	BLT	20–10	18	90	52–38D	1033	CH	BLT	6	1	7	61S
1017	CH	BBRT14	BLT	34–11	26	219	50–36D	1034	BR	BLT	0	0	0	66S
1018	BR	BBRT14	BLT	0–0	0	0	75–61S	1035	CH	BLT	10	8	37	61S
1019	EN	BBRT14	BLT	6–0	13	19	21–7D	1036	CH	BLT	0	0	0	61S
1015	CH							1037	EN	BLT	10	26	141	34D
1038	CH	BFRT14	BLT	8–0	20	128	29–15D	2091	DU	BLT	3	1	5	75S
1039	DU	BFRT14	BLT	0–0	10	19	23–19D	2092	DU	BLT	10	12	41	58D
1040	CH	BFRT14	BLT	0–0	0	0	75–61S	2093	CH	BLT	10	15	26	31P
1041	CH	BFRT14	BLT	0–0	0	0	75–61S	2094	BR	BLT	12	4	14	41P
1042	CH	BFRT14	BLT	0–0	0	0	75–61S	2095	CH	BLT	8	15	25	52P
1043	BR	BFRT14	BLT	0–19	5	40	75–61S	2096	CH	BLT	10	10	18	56D
2098	CK	BBRT12	BLT	0–0	7	8	75–63S	3164	BR	BLT	18	14	69	41P
2099	CK	BBRT12	BLT	0–0	0	0	75–63S	3165	BR	BLT	8	7	28	48D
3159	BR	BBRT12	BLT	22–15	22	253	38–24D	3166	NW	BLT	17	15	85	63S
3161	NW	BBRT12	BLT	20–0	1	21	75–63S	3168	CH	BLT	28	2	28	52P
3162	CH	BBRT12	BLT	0–3	4	16	75–63S	4521	FL	BLT	1	2	3	62P
3163	CH	BBRT12	BLT	0–0.3	0	.3	75–63S	4522	CH	BLT	0	6	15	61S
4509	CH	BBRT14	BLT	10–6	7	32	46–32M	4523	BR	BLT	0	3	9	61S
4510	CH	BBRT14	BLT	0–0	0	0	75–61S	4524	BR	BLT	3	18	153	47D
4512	FL	BBRT14	BLT	7–4	12	250	73–59M	4525	CH	BLT	22	8	163	63S
4513	BR	BBRT14	BLT	14–0	21	184	31–17P	4526	BR	BLT	0	0	0	61S
4514	BR	BBRT14	BLT	18–0	24	250	44–30D							
5750	NW	FSSC13	BSC	0–0	0	0	75–62S	5751	HI	BSC	0	0	0	68S
5753	CH	FSSC13	BSC	0–0	0	0	81–68S	5754	SA	BSC	0	0	0	78S
5760	EN	FSSC13	BSC	0–0	0	0	91–78S	5758	NW	BSC	0	0	0	81S
5762	CH	FSSC13	BSC	0–0	0	0	99–86S	5759	NW	BSC	1	1	5	82S
6783	FL	FSSC20	BSC	0–0	0	0	82–62S	5763	FL	BSC	0	0	0	58S
6786	CH	FSSC20	BSC	0–300	7	316	36–16D	6787	FL	BSC	0	0	0	62S
6791	CH	FSSC20	BSC	0–12	17	207	58–38D	6788	CH	BSC	19	20	172	38D
6792	CH	FSSC20	BSC	0–220	15	240	42–22D	6795	NW	BSC	0	0	0	62S
6794	NW	FSSC20	BSC	0–220	22	286	41–21D	6796	CH	BSC	0	0	0	61S
7819	NW	FSSC23	BSC	0–0	0	0	90–67S	7830	CH	BSC	0	0	0	74S
7821	BR	FSSC23	BSC	1–2	1	5	97–74S	7831	NW	BSC	146	18	211	48D
7824	FL	FSSC23	BSC	0–0	0	0	85–62S	7834	CH	BSC	110	9	170	44D
7828	CH	FSSC23	BSC	20–0	2	40	85–62S							
7833	NW	FSSC23	BSC	24–0	23	211	52–29D							
8903	NW	BSSC21	BSC	0–0	0	0	98–77S	8910	NW	BSC	30	10	41	49M
8914	EN	BSSC21	BSC	40–0	1	41	99–78S	8915	EN	BSC	0	0	0	72S
8916	FL	BSSC21	BSC	300–0	3	303	66–45S	8922	NW	BSC	0	2	4	82S
8920	BR	BSSC21	BSC	0–2	1	4	99–78S	8925	NW	BSC	415	3	420	79S
8924	NW	BSSC21	BSC	0–0	0	0	99–78S							
Means	41 EXP.			14–20	7.4	79	D–16	38	Control		25	6.3	52	D–16

NOTE: Rab. = rabbit number; 1st digit = Exp. No.; Br = breed; TR-S-I = 1st transplant site and interval in days before 2d transplant; CH = chinchilla; Br = Brown-gray hybrids; EN = English; DU = Dutch; CK = Checker; NW = New Zealand White; FL = Flemish; HI = Himalayan; SA = Sable; BB = whole unfrozen Brown-Pearce tumor; BF = whole frozen Brown-Pearce tumor; FS = sedimented cells from frozen tumor fresh; BS = sedimented cells from fresh unfrozen tumor; RT = right testis; LT = left testis; MF = number of metastatic foci; TT = total tumor, primary and metastatic, in cc.; Days = termination date after 1st and 2d inoculations; D = died from Brown-Pearce tumor; S = survived; P = paralyzed from tumor; M = moribund from tumor; SC = subcutaneous transplantation.

compared to 9–21 days for E 0771 mouse carcinoma transplants of comparable size. Jobling did not state which animals died from the tumor, only that the growths did or did not regress. Those growths which did not regress or stop growing are assumed to have killed the rat. Data on rats reinoculated 60–150 days after the first transplant were not used, since many rats died of the first transplant during this period. Jobling did not state that reinoculation within 60 days after the first transplant was ineffectual in changing host resistance, but we believe his data (his Tables 2, 4, 6) warrant this conclusion when rearranged and analyzed by the χ^2 test.

	Died	Recovered	Total
0–30 days	38	42 (38.8)	80
Controls	55	53 (52.4)	108
30–60 days	62	51 (54.8)	113
Total	155	146 (146)	301

Thus, of 193 rats reinoculated 0–60 days after the first transplant, 93 recovered, compared to 53 recoveries among the 108 control rats receiving one inoculation only. Thus, two transplants within 60 days resulted in the same mortality from the Flexner-Jobling tumor as occurred from a single transplant ($\chi^2 = 1.1$, $N = 2$—not significant).

Rous and Murphy (22) reinoculated 29 fowls with the same fowl tumor in a new site and with a different tumor in another site (chicken tumors I, VII, and XVIII were employed). The same tumor took in two of fourteen that were negative after the first inoculation; in three of eleven, regressing or stationary; and in two of four, with progressing tumor. The differences in the host-tumor reactions between the first and second inoculations with the same tumor were not statistically significant. In contrast, the fourteen fowls without tumors had ten positive growths of a different tumor, as against eight positive growths of the different tumor among the fifteen animals with takes on the first inoculation ($\chi^2 = 6.6$, $n = 1$, $P = 0.01$, significant). This indicates that the chief factors of resistance to one tumor were both natural and different from those to another neoplasm.

DISCUSSION

The inoculation of Brown-Pearce or E 0771 tumor tissue which has been kept frozen for several weeks, or which has been lyophilized, resulted in a marked breakdown of the host's resistance to the growth of the same tumor (1, 8, 24).[1] This is in contrast to the negative effect of a double transplantation of fresh whole tumor tissue or of sedimented cells. Recently, Shear *et al.* demonstrated that the supernatant unfiltered fluid of fresh unfrozen C3H tumor 8352 after prolonged low speed centrifugation contains an XYZ factor in full potency (23). In experiments paralleling those listed in Table 1, the supernatant fluid was poured off the sedimented E 0771 cells and injected (9). The result was a marked breakdown in the resistance of the C57BR/cd and BALB/c mice to the C57BL tumor E 0771, whereas the sedimented cells had no appreciable effect on host resistance.

Many years ago when the XYZ enhancing phenomenon was first observed with the Brown-Pearce tumor, the senior author observed similar results obtained by Jobling with heated emulsions of the Flexner-Jobling tumor (15). In a personal communication Dr. Jobling stated that he had had difficulty repeating the experiments, and several others had the same experience. The experiments of Chambers and Russ (10, 12) and Chambers and Scott (11) with the Jensen rat sarcoma, of Lepper (18), and of Rondoni (19) suffered a similar fate. It was concluded that heat was a poor way to demonstrate an XYZ factor in a given tumor, particularly since our emulsions of the Brown-Pearce tumor (7) seemed thermolabile (55° C., ½ hr.) and Jobling's emulsion thermostable (55° C., ½ hr.). Recently, Snell[2] stated that the lyophilized material from mouse tumor 15091a which can induce the XYZ phenomenon is thermostable (55 C., ½ hr.), whereas we have found the XYZ factor (frozen but not desiccated) in mouse tumor E 0771[3] and that in the Brown-Pearce tumor to be thermolabile (7). It was postulated either that the XYZ factors differ in physical properties or that the thermostability is being affected by the medium or by the method of preparation. Both Jobling and Snell[2] and Kaliss, Jonas, and Avnet (14) used such fine emulsions (homogenates) of tumor that no tumor growth resulted from their injection, and their technics varied only in that the former authors heated for ½ hour at 55°, while the latter lyophilized the material. Consequently, we have tabulated Jobling's experiments with the 301 control rats (singly and doubly inoculated animals), the 28 rats injected with unheated homogenate, and the 106 rats injected with heated (55° C., ½ hr.) homogenates.

	Died	Recovered	Total rats
Controls	155	146 (134.2)	301
Unheated	5	23 (12.5)	28
Heated	81	25 (47.3)	106
Total	241	194	435

[1] G. R. Drysdale, G. L. Ross, H. E. Skipper, and A. E. Casey. Unpublished material.

[2] G. D. Snell, unpublished material.

[3] A. E. Casey, F. M. Schabel, Jr., and J. Gunn. Unpublished material.

Jobling's data indicate that the unheated emulsion set up a state of resistance when injected, only five animals so injected dying with the tumor ($\chi^2 = 21$, $P = 0.01$—significant), some 81 rats treated with heated material dying from the tumor. The injection of unheated emulsions of cytoplasm or of nuclei from the Brown-Pearce tumor prepared in a particular manner (Waring Blendor, citric acid) induced a significant state of resistance in the rabbit,[1] whereas the frozen or lyophilized tumor tissue induced a state of susceptibility (1–5, 8, 17, 23–25).

In the original experiments of Rous (20, 21), it would seem that an XYZ effect occurred with chicken tumor I. This neoplasm would not grow at first in any except a special line of Plymouth Rocks and in only 28 of the first 58 special fowls inoculated. At this point, ten special fowls were injected in one site with fresh tumor tissue and in another site with a supernatant fluid—a homogenate of fresh chicken tumor I tissue: (*a*) centrifuged 5 minutes at 2,800 r.p.m.; (*b*) the upper layers of the supernatant recentrifuged 15 minutes at 3,000 r.p.m.; (*c*) the upper part of the second supernate used for injection. This method is almost identical with that used recently by Shear, Imagawa, Syverton, and Bittner (23). Progressive tumors grew in all the fowls (and in seven grew at the site of injection of supernate). This was a statistically significant enhancement of malignancy over the previous experience of 28 tumors among 58 fowls inoculated ($\chi^2 = 9.2$, $n = 1$, $P = 0.001$—significant). Casey noted that the XYZ effect could be obtained with the Brown-Pearce tumor in rabbits by simultaneous injections of the XYZ material at a different site from the tumor inoculum (1). From a fowl, injected by Rous (21) with the supernate plus tumor (as described above), tumor was excised and then inoculated into ten Plymouth Rocks not of the special line; and in five the tumor grew. The enhanced growth of chicken tumor I (associated with the supernate injection) was therefore transmissible serially, since, of 33 market Plymouth Rocks previously injected, the tumor grew in none ($\chi^2 = 18.9$, $n = 1$, $P = 0.001$—significant).

In another experiment (20, 21), Rous took the upper layer of the first supernate from chicken tumor I, filtered it through a coarse No. 2 Berkefeld filter, and injected nine market Plymouth Rocks with the supernate-filtrate alone (one growth resulted) and twenty market Plymouth Rocks with the supernate-filtrate at one site and the chicken tumor I tissue at another site. Of the seventeen free from intercurrent infection, thirteen died from progressive tumor growth (ten had metastases to distant organs), tumors grew in three and then regressed, and one had no tumors. This compared with 0 tumors among the 33 market Plymouth Rocks previously inoculated with the chicken tumor I alone, a statistically significant increase in malignancy compatible with the XYZ effect ($\chi^2 = 34$, $n = 1$, $P = 0.0001$—significant).

Of interest, therefore, are the observations that, with four malignant tumors in four animal species (the Brown-Pearce tumor of rabbits, E 0771 carcinoma of mice, chicken tumor I of fowls, and the Flexner-Jobling tumor of rats), reinoculation with the same tumor does not alter the host's reaction to the neoplasm, although potent XYZ factors are presumably present in the tumor emulsion.

SUMMARY

Mouse mammary carcinoma E 0771 of C57BL/6 origin, known to yield XYZ factors, was inoculated into 30 C57BN/cd as well as 30 BALB/c mice. Eight to 15 days later these animals, as well as an additional 30 C57BR/cd and 76 BALB/c mice, received an injection of viable E 0771 tumor cells. No significant difference in tumor development was noted when animals receiving only a single injection of E 0771 tumor cells were compared to mice receiving a double inoculation of the same tumor.

Similarly, the prior inoculation of fresh Brown-Pearce rabbit epithelioma cells failed to alter the malignant process in recipient hosts receiving a second implant of the same tumor administered at a different site 14–21 days later. This was made known in a series of experiments utilizing 78 rabbits of mixed breeds. Of these animals, 40 served as recipients of a double inoculum of viable Brown-Pearce tumor cells, while the remainder received only a single injection and served as controls. These results are in sharp contrast to the XYZ phenomenon observed when frozen Brown-Pearce tumor tissue is given prior to challenge transplantation with fresh homologous tumor tissue.

Thus, for these two mammalian tumors, the E 0771 mammary carcinoma of mice and the Brown-Pearce epithelioma of rabbits, the inoculation of test animals with fresh viable cancerous tissue, followed by a second implantation of the same tumor, does not alter significantly the malignant process in the recipient hosts, although potent XYZ factors are known to be demonstrable when these tumors are subjected to prolonged frozen storage, lyophilization, or centrifugation.

That this phenomenon may be applicable to

other experimental neoplasms is made apparent when the data of Jobling and Flexner concerning a rat carcinoma and the data of Rous and Murphy based on experiments with a fowl tumor are reviewed. Although the prior injection of heated rat carcinoma cells resulted in an enhancement of a subsequent transplant of tumor cells, the prior injection of fresh unheated tumor cells did not significantly alter the progress of a subsequent transplant with homologous cancer tissue. Similarly, although the Rous fowl sarcoma appears capable of exhibiting the XYZ phenomenon when supernatant fluid derived from the tumor is utilized, no enhancement of tumor transplant growth occurred when an injection of viable Rous tumor cells preceded challenge with the same tumor tissue.

CONCLUSIONS

The prior inoculation of homologous fresh whole tumor cells, known to yield demonstrable XYZ factors, did not significantly alter the incidence, growth, metastases, or mortality upon subsequent reinoculation of the E 0771 mammary tumor of mice and the Brown-Pearce tumor of rabbits.

These results present a sharp contrast to the significant enhancement in the course of the malignant disease when XYZ factors, obtained by subjecting the cancerous tissue to freezing, lyophilization, or centrifugation are injected prior to implantation of the corresponding tumor.

A review of the literature reveals that similar results were obtained when a rat carcinoma and a fowl sarcoma were utilized experimentally in their respective hosts.

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ADDENDUM

Since this paper was submitted for publication, statistical analyses have been made of papers by Cloudman (Cloudman, A. M. Successful Interspecies Transplantation of a Mouse Tumor. Science, **76**:525–26, 1932) and by Lewis and Lichtenstein (Lewis, M. R., and Lichtenstein, E. G. Breaking Down

the Resistance of Albino Mice to the Transplantation of Tumors Induced by 1:2:5:6: Dibenzanthracene in a Different Strain of Albino Mice. Am. J. Cancer, **27**:246–56, 1936; Lewis, M. R., and Lichtenstein, E. G. Further Studies on the Breaking Down of Resistance of Mice of One Strain to the Transplantation of Tumors of Mice of Another Strain. Am. J. Cancer, **28**:746–51, 1936) which bear on the subject of reinoculation with the same tumor. Cloudman transplanted Strain A mammary carcinoma 15091a (which was fatal in each of 150 strain A mice inoculated) into 425 mice of six foreign strains, the negative or recovered animals being reinoculated with the same tumor. Tumor 15091a killed 50 of 85 MD mice (W. S. Murray's dilute brown), 63 of 104 D mice (Strong's dilute brown), ten of 21 B mice (Little's blacks), seven of 46 Ld (J. M. Murray's leaden), one of 78 Z mice (Zavadskaia's albinos), three of 91 Mus bactrianus on the first inoculations; and 26 of 85 MD, 28 of 41 D, two of ten B, 0 of 39 Ld, 0 of 77 Z, and 0 of eleven Mus bact. negative or recovered mice on the second transplantation. The only statistically significant difference was in fewer reinoculation takes in the Ld strain, a strain naturally resistant on the first inoculation ($X^2 = 6.4$, $n = 1$, $P = 0.01$). In other words, "acquired resistance" could only be demonstrated in a strain with a high natural resistance to the neoplasm; or, it might be reasoned that high natural resistance could be even better demonstrated by reinoculation. Mice with little or no natural resistance showed no significant change in reaction upon reinoculation.

Lewis and Lichtenstein inoculated twenty C3H mice with BALB tumors Nos. 1, 3, and 6. From the 60 first transplants ten progressive tumors resulted; upon second inoculation of the same twenty mice with the same three tumors, thirteen of 60 transplants grew; with third and fourth inoculations of the same mice with the same tumors, twelve of 65 transplants grew (not sig.). The same authors inoculated 24 BALB mice with A strain tumors 9, 14, and 20: twelve growths occurred among the first 58 transplants; with the second inoculation of the same tumors, eighteen of 51 transplants grew; with the third and fourth inoculations of the same mice with the same tumors, there were nineteen growths in 62 transplants (not sig.). Again, 48 BALB mice were inoculated with C3H tumors 4, 8, 12, 13, 14, and 28; in the first inoculation one of 148 transplants grew; with the second inoculation two of 148 transplant grew; with the third and fourth inoculations five of 204 transplants grew (not sig.). Combining the three series, there were 23 tumors among 266 first inoculations with a given tumor; 33 of 259 second inoculations of the same tumors into the same mice; 36 of 331 third and fourth inoculations (differences not sig.).

Lewis and Lichtenstein attacked the problem of whether multiple inoculations of C3H tissue (C3H tumors) into BALB mice, or of BALB tissues (BALB tumors) into C3H mice, or of A tissues (A strain tumors) into BALB mice could break down the resistance of the mice of the foreign strain to the growth of inbred strain tumors. Their data were difficult to tabulate, but some 110 mice seem to have received 8–20 trocar implants (usually at 4–6-day intervals) of some 25 foreign mouse strain tumors. From the first seven inoculations (over a 30-day period) into each mouse 45 progressive tumors developed among 794 transplants; from the eighth to the twentieth inoculation (during second to fourth month) into the same mice 65 tumors developed among 801 transplants; the difference was probably significant in that more tumors occurred in the second series than in the first ($X^2 = 3.9$, $n = 1$, $P = 0.05$). Analysis revealed, however, that some tumors used in the first seven inoculations were not employed in the second series (Strain A tumors 4, 6, 7, 10, 11, 13; BALB tumors 5 and 7), and, vice versa, some tumors employed in the second series had not been employed in the first series (C3H tumors 11 and 25; BALB tumor 9). Thus, there were six growths among 291 transplants of tumors used only in the first seven inoculations and fourteen growths among 74 transplants of tumors used in the eighth to twentieth inoculation but not employed in the first to seventh inoculation; this difference was significant ($X^2 = 32.4$, $n = 1$, $P = 0.01-$). As a further check on whether the observed increased incidence in the eighth to twentieth inoculation was due to the use of new tumors, an analysis was made including only tumors which were used in both the first to seventh and eighth to twentieth series. There were 39 growths among 503 transplants in the first as compared to 51 among 711 transplants in the second series of inoculations when the same tumors were used in both series; there were not only no significant differences, but the values were so nearly alike for the tumor incidence in the first as compared to the second series as to approach positive proof that no such differences existed ($X^2 = 0.144$, $n = 1$). In summary, it may be concluded that the data of Lewis and Lichtenstein (not the conclusions of the authors) show that seven to twenty transplantations of living BALB tumor tissues into C3H mice or C3H and A tumor tissues into BALB mice did not significantly break the resistance of the mice to either the same tumor or to different tumors of the same strain.

Blood Volume, Body Water, and Circulation Time in Patients with Advanced Neoplastic Diseases*

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Progressive growth of a neoplasm is dependent upon the development of additional vascular channels through which the tumor derives its nutrients. Compression and invasion of lymphatics and blood vessels by neoplastic growth may produce edema and may bring about other physiologic abnormalities in the content and the dynamics of intra- and extravascular fluid. There is a paucity of clinical studies directed toward the determination of fluid compartments in patients with cancer. Rountree and Brown (6), using the Congo-red dilution method, observed that the blood volume was increased in eight of ten patients with myelocytic leukemia and in two of four patients with lymphatic leukemia. Recently, Furth and Sobel (4) reported hypervolemia in mice that received transplants of granulosa-cell tumors. Storey, Wish, and Furth (8), using radioactive technics, determined blood volume in mice bearing transplantable mammary carcinoma as well as other tumors, and found an increase in the total blood volume, which was most evident as an increase in the plasma volume. The tumors themselves, however, were oligemic.

The present report deals with an exploratory study of fluid compartments in 33 patients with a wide variety of advanced neoplastic disease. Emphasis was placed upon the determination of a number of values as simultaneously as possible in order to reveal possible correlations between the findings.

SUBJECTS

Forty-seven studies were carried out on 33 patients, as listed in Table 1. The diagnosis in all cases was substantiated by at least one relevant biopsy. All patients had advanced, metastatic neoplastic diseases. They were, however, in relatively good physical status at the initial study, and only one patient (Deq) was cachectic. Twenty-four of the patients have died, and autopsies were available for all; nine patients are still alive.

The patients were hospitalized and observed for approximately 1 week prior to the study. Only cases with normal cardiac function, including normal electrocardiograms, and without evidence of renal impairment were selected. The initial study was performed at least 1 month following any previous treatment by surgery or radiation. Studies on patients were repeated 1–4 months following the initial determinations. Progression of the disease or its palliation by treatment afforded an opportunity to study patients in different stages of the disease.

The patients were studied after bed rest and fasting overnight. Venous blood was sampled for the necessary chemical and hematological examinations. Heparin was used as the anticoagulant for all samples other than the hemogram, which was collected in oxalate.

PROCEDURE

Exactly 1.0 gram of antipyrine was administered intravenously. The patients were then allowed to proceed with the usual ward routine, including meals and fluids. One, 2, 3, 5, and 7 hours following the administration of antipyrine, venous samples were withdrawn. A Millikan oximeter earpiece was attached to the left pinna for warming. A blood sample was obtained for packed cell volume determination and for a blank for the plasma volume. The lung-to-ear circulation time was determined at varying hypoxic blood oxygen saturations. The patients next received 45 mg. of Evans' Blue, T1824, diluted in 10 cc. of fluid, in five 2-cc. aliquots over a period not to exceed 3 minutes, for the determination of arm-to-ear circulation time. Twenty minutes later venous blood was drawn from the opposite arm for the determination of the plasma volume.

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TABLE 1

Blood Volume, Body Water, and Circulation Time in Patients with Advanced Neoplastic Diseases

Name	Sex	Age	Diagnosis	Height (cm.)	Weight (kg.)	Hemoglobin (gm. per cent)	Packed cell volume (cu. mm.)	Total protein (gm. per cent)	Albumin (gm. per cent)	Edema	Basal metabolic rate	Blood volume (l.)	Blood volume (cc/kg)	Plasma volume (l.)	Plasma volume (cc/kg)	Body water (l.)	Body water (per cent body weight)	Arm-to-ear circulation time (seconds)	Lung-to-ear circulation time (seconds)
1. Kup.	F	60	Carcinoma, breast	157	70.2	12.7	38	8.3	3.8	0	+12	5.6	79	3.5	50	32.4	46.2	6.0	4.1
2. Fra.	F	51	Carcinoma, breast	168	67.0	13.5	36	5.2	1.9	++	− 8	4.4	66	2.8	43	31.2	46.5	9.8	6.3
3. Riv.	F	57	Carcinoma, breast	152	53.0	13.1	39	8.0	4.1	0	− 9	4.3	81	2.6	49	24.3	46.4	12.2	6.1
4. Cla.	F	59	Carcinoma, breast	154	56.6	12.5		6.5	3.7	++						29.5	52.1	11.8	5.5
5. Urk.	M	31	Carcinoma, testes	178	65.4	11.6	35	5.7	2.9	0	+24	5.5	84	3.6	55	36.2	52.2	9.1	4.1
6. Urk.	M	31		178	62.8	9.5	30	6.5	3.4	+	+19	5.2	83	3.6	58	36.5	58.1	9.9	5.0
7. Urk.	M	31		178	55.2	5.6	20	5.9	3.8	++++		6.0	110	4.9	88	31.3	56.7	12.0	7.0
8. Yeo.	F	35	Carcinoma, cervix	167	53.8	11.7	39	7.0	3.1	0		4.9	91	3.0	55				
9. Bor.	M	42	Carcinoma, lung	168	52.5	13.2	45	6.6	3.1	0		7.6	146	4.2	80	35.1	67.0	11.6	5.9
10. Fey.	M	70	Carcinoma, lip	176	58.6	10.0	37	5.9	2.5	0		5.2	108	3.3	68	29.2	60.0		
11. Mah.	M	60	Carcinoma, nasopharynx	175	57.9	11.2	38	6.8	3.4	0		5.6	98	3.5	60				
12. Har.	F	38	Melanoma	173	64.5	15.8	40	6.0	3.3	+	+ 6	4.6	71	2.7	42	34.1	52.5	12.7	5.2
13. Har.	F	38		173	60.5	12.0	39	6.2	3.7	+	+12	5.0	84	3.1	51	28.9	47.8	10.7	4.3
14. Har.	F	38		173	59.4	11.1	38	5.8	2.9	++	+ 8	3.9	66	2.4	41	27.9	47.9	9.0	3.8
15. Har.	F	38		173	61.1	12.7	32	5.9	3.6	++++	+23	6.2	102	4.2	69	45.1	73.8	8.7	4.7
16. Top.	M	28	Melanoma	177	66.6	10.5	33	5.8	2.9	++		7.0	105	4.6	70				
17. Sav.	M	71	Melanoma	170	55.5	10.8	22	7.4	3.1	0		5.5	100	3.3	60				
18. Geo.	F	45	Multiple myeloma	154	60.7	13.8	30	6.9	3.6	0		3.3	55	2.3	39	24.8	40.8	7.0	4.3
19. Geo.	F	45		154	62.3	13.1	30	6.8	4.2	0	− 3	2.9	48	2.0	33	25.4	40.8	8.3	5.8
20. Cli.	F	55	Multiple myeloma	160	63.2	10.0	22	7.4	3.1	0		4.4	70	3.5	55				
21. Cli.	F	55		160	62.0	9.5	31			0		4.5	72	3.0	49				
22. Pit.	M	58	Mycosis fungoides	178	68.9	10.8	35	5.9	2.9	0	− 5	5.6	81	3.7	54	36.2	52.5	11.7	5.5
23. Can.	M	50	Lymphosarcoma	173	71.5	14.9	44	7.1	4.1	0	+10	6.8	95	3.8	53	43.8	61.3	14.8	5.9
24. Deq.	F	24	Hodgkin's disease	161	38.4	10.0				++						24.6	60.2	8.2	5.1
25. Deq.	F	24		161	39.6	9.2	29	4.5	2.1	++		4.5	114	3.2	81	27.4	69.2	8.5	5.0
26. Deq.	F	24		161	38.8	7.6	22	5.6	2.0	++		4.7	122	3.7	95	27.1	69.9	8.1	4.8
27. Scr.	F	27	Hodgkin's disease	169	53.4	5.7	31	7.7	3.5	0	+26	5.3	100	3.7	69	21.9	41.0	9.6	4.5
28. Scr.	F	27		169	52.7	15.5	47	7.1	3.4	0		4.7	89	2.5	47	24.3	46.4	12.4	6.9
29. Kin.	M	13	Hodgkin's disease	169	49.4	14.3	40	7.3	3.5	0	+ 6	6.1	123	3.7	74	29.9	60.0	11.8	5.5
30. Kin.	M	13		169	56.4	13.3	39	6.4	4.0	0	+ 2	5.9	106	3.6	65	42.2	74.4	6.9	4.7
31. Kir.	M	35	Lymphatic leukemia	168	70.0	8.1	40	5.3	3.1	++		5.3	77	3.2	46				
32. Flu.	F	79	Lymphatic leukemia	138	59.2	9.5	34	5.3	2.7	0		7.0	118	4.6	78				
33. Elm.	M	70	Lymphatic leukemia	168	50.0	9.1	33	3.8	2.4	0		6.5	130	4.3	87				
34. Val.	F	68	Lymphatic leukemia	159	50.0	10.0	35	4.5	2.3	0		5.6	113	3.6	74				
35. And.	M	14	Lymphatic leukemia	170	55.6	10.3	37	7.0	3.8	0		6.8	123	4.3	78	27.7	49.8	7.5	5.2
36. Lar.	M	36	Lymphocytic leukemia	175	56.0	12.1	38	6.4	4.0	0	+42	5.2	94	3.2	57	32.5	58.0	19.2	6.4
37. Joh.	M	51	Lymphocytic leukemia	175	70.0	10.1	30	6.6	2.9	0	+26	7.2	103	5.0	72	41.2	58.9	12.9	6.3
38. Lew.	M	31	Lymphocytic leukemia	148	62.6	9.2	43	6.1	2.6	+	+64	6.1	98	3.5	56	41.0	69.0	8.9	5.0
39. Lew.	M	31		148	56.3	10.1	37	4.7	2.8	+++		7.2	128	4.5	81	43.6	77.4	9.4	4.1
40. Fee.	F	46	Myelocytic leukemia	160	47.3	9.4	35	6.0	3.4	0		4.9	104	3.2	68				
41. Tra.	M	72	Myelocytic leukemia	170	48.3	8.1	11			0		5.0	104	4.5	93				
42. Her.	M	51	Myelocytic leukemia	177	101.7	5.9	20	5.2	3.2	0		7.8	77	6.2	61				
43. May.	F	28	Myelocytic leukemia	149	49.7	5.0	17	7.3	3.5	0		2.6	53	2.2	44	25.5	51.3	6.6	3.6
44. May.	F	28		150	43.5	8.0	45	7.4	2.5	0						28.5	66.0	9.7	6.3
45. Wal.	M	37	Myelocytic leukemia	180	64.0	8.0	30	6.1	3.2	0	+ 5					43.1	67.4	10.0	6.0
46. Wal.	M	37		180	61.9	8.8	40	6.1	3.2	0	+17	7.4	120	4.4	72	29.9	48.3	11.6	6.2
47. Coo.	M	49	Myelocytic leukemia	178	69.7	5.6	20	6.1	2.8	0	+43	8.7	125	7.0	100	30.9	44.3	11.1	5.4
Low		13		138	38.4	4.6	11	3.8	1.9			2.6	48	2.1	33	21.9	40.8	6.0	3.6
High		79		180	101.7	15.8	47	8.3	4.2			8.7	146	7.0	100	45.1	77.4	19.2	7.0
Average		42		166	58.6	10.54	34	6.3	3.2			5.54	95.7	3.66	63.2	32.08	56.3	10.2	5.29

Blood volume was calculated from the plasma volume and the packed cell volume as determined by means of the Wintrobe hematocrit tube after 20 minutes of centrifugation. No correction for trapped plasma was made, but in patients with leukemia with high leukocyte counts the amount of dye in the laked white cells was determined and was found to be negligible.

The total body water was determined by the antipyrine method described by Brodie *et al.* (2) and by Soberman *et al.* (7). The arm-to-ear circulation time and the lung-to-ear circulation time, as measured by the oximeter, were determined by the methods described by Bierman (1).

Serum proteins were determined by the micro-Kjeldahl method. Hemoglobin was measured spectrophotometrically. The erythrocyte and leukocyte counts were made with NBS certified hemocytometers and Trenner automatic-filling pipettes.

The normal values for the determinations in this study are as follows: total body water, 39–61 per cent of the body weight, or 53.0 ± 1.37[1] per cent; plasma volume, 42–59 cc/kg body weight, for a mean of 45 ± 4.0 cc/kg; and blood volume, 72–99 cc/kg, for a mean of 85 ± 8.9 cc/kg of body weight. The arm-to-ear circulation time is 9.6–19.9 seconds, for a mean of 13.2 ± 0.45 seconds, and the lung-to-ear circulation time is 4.8–7.1 seconds, for a mean of 5.7 ± 0.29 seconds.

RESULTS

Six normal adults were studied in the same manner as the patients with neoplastic disease. The values fell well within the ranges for normal individuals given above.

The data on 47 studies performed in 33 patients are detailed in Table 1. Analyses of the total data were carried out on any one determination, using re-studies on patients on the same basis as initial studies and for possible correlations between various determinations.

Total body water.—The total body water as determined by the antipyrine method was within the normal range except in patients with clinical evidence of edema, half of whom showed serum albumin levels of less than 3.0 gm/100 cc. In 21 instances in patients without edema the total body water was 53.9 ± 2.14 per cent of body weight. In thirteen determinations on six patients with edema, the total body water was 60.0 ± 3.0 per cent. The development of edema is reflected in a significant ($P < 0.03$) increase in the total body water.

Plasma and blood volume.—The plasma volumes were found to be increased significantly ($P < 0.01$) in patients with neoplastic diseases. The over-all mean of the plasma volume, in 43 determinations, was 63.2 ± 2.5 cc/kg, as compared to the normal 45 ± 4.0. In contrast, the mean value of the blood volume was 95.7 ± 3.4 cc/kg, which was not significantly different from the normal of 85 ± 8.9.

In sixteen determinations in patients with a packed cell volume of over 38, the blood volume ranged from 79 to 146, with the mean at 94.9 cc/kg. In fourteen determinations with a packed cell volume between 31 and 37, the blood volume ranged from 66.1 to 130, with the mean at 102.4. In thirteen determinations with a packed cell volume of 30 or less, the blood volume ranged from 48 to 125, with a mean of 89.5. A reduction in the packed cell volume, therefore, was not the only reason for the apparent increase in the blood volume. A similar lack of correlation was observed between plasma or blood volume and blood proteins.

The data showed only two values for plasma volume beneath the lower limit of normal of 42 cc/kg, both in a single patient with multiple myeloma. In contrast, 21 of 43 determinations of plasma volume were above the normal limit of 60 cc/kg.

In relation to total blood volume, seven of the determinations were beneath the lower limit of normal of 72 cc/kg; four of these occurred in the two patients with multiple myeloma and one (May) in a patient with myelocytic leukemia who had had a severe hemorrhage 2 weeks before the study. The next lowest blood volume, of 77 cc/kg, in a patient with myelocytic leukemia (Her) was also determined within a week after a severe renal hemorrhage. In contrast, 19 of 43 determinations were above the upper limit of normal of 100 cc/kg. This occurred in five of twelve patients with carcinoma or melanoma, three of four patients with Hodgkin's disease, and ten of fourteen patients with leukemia. Thus, in over half of the patients with neoplastic diseases increased blood and plasma volumes were noted. This increase was not accountable on the basis of decreased packed cell volume, blood proteins, or the presence of edema.

It appears that a considerable proportion of patients with advanced neoplastic disease demonstrated a relative hypervolemia, particularly in the plasma volume.[2]

Circulation time.—The circulation time in patients was generally decreased as compared to

[1] The standard error of the mean is used throughout.

[2] Through the courtesy of Dr. S. P. Masouredis, plasma volumes in cases Nos. 10, 11, 17, 21, and 42 were measured with human serum albumin labeled with Iodine^{131}, simultaneously with T1824. The plasma volumes were, respectively, 2.8, 3.8, 2.9, 3.1, and 6.1 liters, the maximum difference being 0.4 l., the averages differing only by 0.1 l.

that of normal individuals. The arm-to-ear circulation was from 6.0 to 19.2, for a mean of 10.2 ± 0.46 seconds in 33 determinations, a value significantly different ($P < 0.01$) from the normal mean of 13.2 ± 0.45. The lung-to-ear circulation time was from 4.1 to 7.0 seconds, with a mean of 5.3 ± 0.16 seconds, not significantly different from the normal.

Of thirteen determinations in patients with normal hemoglobin, red blood cell count, and blood proteins, the circulation time was 11.5. There were no consistent relationships between blood volume and plasma volume and circulation time.

It is concluded that circulation time is more rapid in patients with advanced neoplastic disease than in normal individuals, a finding not necessarily related to their state of anemia.

DISCUSSION

The data obtained in this preliminary exploration of the fluid compartments and the physiological dynamics in patients with advanced neoplastic diseases reveal a number of interesting and possibly significant findings. Patients with a wide variety of neoplastic diseases demonstrate (*a*) total body water in the normal range, unless clinical evidence of edema was present, (*b*) increased plasma volume and increased blood volume, and (*c*) rapid circulation time. These findings were not mathematically interrelated, nor could they be correlated with hemoglobin, packed cell volume, or blood proteins. No significant differences or correlations were ascertained when the data were related to the surface area of the individuals. It must be kept in mind, however, that Tanner (9) has pointed out the fallacy of spurious correlation of measurements to weight and to surface area standards.

It is evident that the measurements are only as reliable as the particular methods that were employed. The dye dilution technic, with the use of T1824 or other materials, has been rightfully criticized as an accurate measure of plasma and blood volume (3, 6). Antipyrine is not an ideal substance to use in the measurement of body water, as Soberman *et al.* (7) point out. In addition, it cannot be assumed without reservation that findings on normal individuals can be compared directly to findings on patients, in whom loss of weight, increased capillary permeability, and other abnormal factors may alter the results of any determination.

The presence of relative hypervolemia, in terms of over 100 cc/kg of body weight, in over half of the patients with advanced neoplastic disease requires a partial interpretation of the anemia seen in patients with far advanced cancer and similar debilitating conditions. The anemia may well reflect, in part, a dilution of hemoglobin and erythrocytes in an increased volume of plasma not readily measured by such means as are available at the present time. It should be emphasized that there are no implications that the findings of this study are in any way specific for neoplastic diseases, or, for that matter, for patients with early or less advanced neoplastic diseases.

SUMMARY

In 33 patients with far advanced neoplastic diseases, 47 studies of fluid compartments were performed, utilizing measurements of total body water by the antipyrine method, plasma and blood volume by the T1824 method, and circulation time from arm-to-ear and lung-to-ear by a modification of the oximeter-dye technic.

Though the studies were performed as nearly simultaneously as possible, no correlations could be made between the measurements obtained. A relative hypervolemia was observed in the majority of the fourteen patients with leukemia. The circulation times appeared to be more rapid in the patients studied than in normal individuals. Body water was observed to be normal except in the presence of clinical edema, no unusual variations being related to the severity of the diseases present.

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The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia

I. Glycolysis and Respiration*

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For almost half a century, there has been a durable interest in the intermediary metabolism of leukocytes, no doubt because leukocytes have certain unique attributes. Their uniform dispersion in a fluid medium permits ready availability and accurate enumeration, and their involvement in leukemia makes possible an investigative approach to this human disease within the area of cellular biochemistry.

Striking biochemical differences between the leukocytes of health and leukemia have been shown, differences not attributable solely to cell immaturity. For example, the leukocytes of chronic myelocytic leukemia are high in histamine (21) and low in alkaline phosphatase (20), while the leukocytes in the disease polycythemia vera, which has passed morphologically into the so-called "leukemic" stage, show high alkaline phosphatase levels (5), the converse of the picture in true leukemia.

Many studies have been published on the glycolytic and respiratory metabolism of leukocytes. Much of this work is difficult to interpret because of variations in experimental procedure. It has been variously held that leukocytes evinced a metabolic pattern typical of cancer cells, according to the classification designated by Warburg (23), with moderate respiration and high aerobic glycolysis (2) and, conversely, that leukocyte metabolism resembled that of embryonic tissue (10) with low aerobic glycolysis. Authors have differed also on the question whether, in these metabolic activities, the normal leukocyte differed from the leukemic. No studies have been reported in which the incubation medium has been carefully controlled with regard to added substrates and coenzymes.

* Based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

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Advances in the technics for isolating leukocytes from whole blood (7, 14, 18, 22) invite a detailed reconsideration of this problem in the light of newer technical knowledge. It is the purpose of this paper to present new data on the oxygen consumption, glucose utilization and lactic acid production of normal and abnormal leukocyte homogenates measured in a system containing glucose and hexose diphosphate as substrates and fortified with various coenzymes. Experiments to be published in a companion paper (6) will indicate that results show great variation depending on the incubation mixture used. It will be seen that, although its basic reaction patterns resemble those of other tissues, the leukocyte has certain unusual and interesting biochemical characteristics. Clear-cut differences between normal and leukemic and between chronic myelocytic and chronic lymphatic leukemic leukocytes will be presented and discussed.

MATERIALS AND METHODS

Leukocytes were isolated from the blood of normal and leukemic individuals, homogenized and incubated in the conventional Warburg apparatus to determine O_2 consumption. Following incubation, lactic acid was determined in an aliquot of the incubation mixture, and residual glucose was measured in the remaining flask contents after heat inactivation of the tissue enzymes.

Clinical material.—Normal individuals were laboratory personnel who were in good health. Leukocyte counts of normal individuals were all between 5,000 and 10,000/c. mm. with normal differentials. The patients with chronic myelocytic and lymphatic leukemia were hospitalized. Some were receiving radiation therapy. In all cases diagnoses were based on bone marrow examinations in addition to clinical findings. This paper includes data obtained in 39 experiments on twenty normal subjects, 23 experiments on nine patients with

chronic myelocytic leukemia, and ten experiments on seven patients with chronic lymphatic leukemia. The patients with chronic myelocytic leukemia had leukocyte counts averaging 106,500/c. mm. with a range of 26,800–394,000. All differentials showed marked shifts to the left with myelocytes, metamyelocytes, band forms, and mature neutrophiles predominating. The patients with chronic lymphatic leukemia had counts ranging from 21,650 to 297,800 with a mean of 124,100. The average lymphocyte percentage was 93 per cent (range, 78–99 per cent).

Isolation of leukocytes.—The separation of leukocytes from whole blood was in most cases accomplished by mixing venous blood with a freshly prepared solution of bovine fibrinogen (Fraction I), Armour. The fibrinogen, diluted in an isotonic (1.15 per cent) solution of KCl adjusted to pH 8.4 with 0.04 M $KHCO_3$, was of such concentration that the packed red cell volume of the final mixture was approximately 30 volumes per cent, and its fibrinogen content 7 mg/ml (7). The citrate content of the fibrinogen was high enough to function adequately as an anticoagulant. Volumes of blood specimens varied from 20 to 100 ml. Leukocyte and differential counts were made on capillary blood preparations.

All foam was carefully removed from the mixture before it was allowed to stand. Most of the red cells had sedimented within 15 minutes, and great care was exercised to permit the sedimentation tubes to remain at room temperature no longer than necessary. In no case did the red cell sedimentation require more than 20 minutes. The supernatant plasma–fibrinogen suspension of leukocytes and platelets was pipetted quickly into ice-cold 50-ml. centrifuge tubes containing 20 ml. of cold alkaline isotonic KCl solution.

In a number of experiments, leukocytes were isolated by means of a phyto-hemagglutinin from red beans (kindly provided by Dr. D. A. Rigas) according to the technic of Li and Osgood (14). Venous blood was mixed with a solution containing 1 mg of phytohemagglutinin/ml and a small amount of crystalline heparin. 0.2 Ml. of this solution was used per 10 milliliters of blood. Centrifugation at 300 r.p.m. (in an International centrifuge size 2, type V with No. 240 head) for 2 minutes yielded a maximum plasma volume containing a uniform suspension of leukocytes with no "buffy layer."

Preparation of homogenates.—The leukocyte suspension, kept chilled during all subsequent handling, was centrifuged at 4° C. for 4 minutes at 1,000 r.p.m. The supernatant liquid containing most of the platelets was discarded. The leukocytes were resuspended in 50 ml. of ice-cold KCl solution and recentrifuged, the washing being repeated twice. The final suspension was adjusted to a volume of 10 ml., and six cell counts were done. Prior to homogenization, the volume of the suspension, containing a known number of leukocytes, was concentrated to 4 ml., a volume convenient for the homogenizers used. Homogenization was carried out in the all-glass Potter-Elvehjem homogenizer in the cold for a period of 4 minutes. Estimates were made of the success of homogenization by microscopic inspection. In practically all instances it appeared that about 25 per cent of the cells remained intact. Further homogenization to reduce this number seemed to reduce considerably the level of oxygen consumption, and it was arbitrarily decided to accept the homogenate, although a number of cells were not disrupted. In several experiments, homogenization was omitted. Prior to pipetting the homogenate into the Warburg flasks, its volume was adjusted to the desired level by adding cold diluent. Care was taken to wash down the homogenizer pestle to preserve the accuracy of the cell counts done prior to homogenization.

Manometric technic.—Cold homogenate was pipetted into the main portion of the Warburg flasks which had been previously prepared and kept chilled. 0.2 Ml. of 20 per cent KOH was placed in the center well with a filter paper wick. Air atmosphere was used throughout, because preliminary experiments with oxygen did not yield higher oxygen uptakes. The flasks were placed in the bath and equilibrated at 38° C. for 5 minutes. The cocks were then closed and readings commenced. All experiments were conducted in duplicate or triplicate.

Reagents.—The system employed in most experiments was as described in Table 1 (this is referred to below as the "complete system").

TABLE 1

COMPOSITION OF INCUBATION MIXTURE

Addition	Amt. stock soln. used (ml.)	Conc. of stock soln. (M/l)	Final conc. (M/l)	Amt. in incubation mixture (μM)
Phosphate buffer, pH 7.4	0.15	0.187	0.01	28.0
ATP-Na*	0.3	0.01	0.0011	3.0
DPN†	0.2	0.01	0.0007	2.0
Cytochrome c‡	0.4	0.0001	4×10^{-5}	0.04
Glucose	0.3	0.05	0.0053	15.0
HDP†	0.15	0.1	0.0053	15.0
$MgCl_2$	0.15	0.1	0.0053	15.0
H_2O	0.60			
Homogenate	0.55			

Total vol. of reaction mixture, 2.8 ml.

Abbreviations used: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; HDP, hexose diphosphate (fructose 1,6-diphosphate).

* Purchased from Pabst Laboratories, Milwaukee, Wis.

† Purchased from Schwarz Laboratories, New York, N.Y.

‡ Purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Stock solutions of commercial preparations of disodium ATP, DPN, cytochrome c, and HDP were neutralized and kept frozen. Weekly assays were performed to insure stability: ATP was determined by E_{260} measurements of adenine (19) and by inorganic phosphorus determinations (9); DPN by the E_{340} method following reduction with sodium hydrosulfite using an E_m (1 cm.) value of 6.22×10^6; (11) cytochrome c by E_{550} measurements using E_m (1 cm.) values of 0.9×10^7 (oxid.) and 2.81×10^7 (red.) (19); and HDP by fructose (16) and inorganic phosphorus determinations.

Analytical methods.—Glucose was determined by various methods including the anthrone method (15), the ferricyanide method of Kingsley and Rheinhold (13), and a modification (4) of the glucose oxidase method of Keilin and Hartree (12). The latter proved far the most satisfactory and specific. At the end of the experimental incubation period, the flasks were inactivated by immersion in a water bath at 80° C. for 3 min. Aliquots (0.3 ml.) were taken for the lactic acid determination. 0.3 Ml. of glucose oxidase solution,[1] 0.1 per cent in phosphate buffer (of composition calculated to produce a final solution of pH 5.9 and 0.1 M after tip-in), and 0.1 ml. of catalase[2] solution were placed in the side-arm of the Warburg flask. Flasks were al-

[1] Obtained through the courtesy of E. M. Weber, Chas. Pfizer & Co., Inc.

[2] Purchased from Vita-Zyme Corp., Chicago, Ill. The solution provided commercially, assayed directly by the method of von Euler and Josephson (8), had a Kat. f. value of 35 per ml.

lowed to reach temperature equilibrium at 38° C., cocks were closed, and the reaction timed from the moment side-arm contents were tipped in. Residual glucose was oxidized completely to gluconic acid, permitting calculation of glucose content from O_2 consumption. Appropriate sample blanks and reference standards were run with each experiment. Glucose disappearance was taken to be equal to initial glucose concentration minus residual glucose. The glucose oxidase used had high specificity, failing completely to oxidize glucose-6-phosphate or other of the intermediates of carbohydrate metabolism. In this method, the mere phosphorylation of glucose is registered as glucose utilization.

Lactic acid was determined by the method of Barker and Summerson (3), with lithium lactate as reference standard.

Homogenates were used in all experiments, because it was desired to fortify the system with coenzymes and to include substrates which are reported to pass through cell membranes with difficulty. In preliminary experiments homogenates consistently gave higher and more prolonged activity than intact cells. Homogenate activity remained linear with time up to three hours and was proportional to the amount of homogenate incubated as shown in Chart 1. This problem was not explored at extreme ranges of cell concentration but only in the vicinity of the concentrations being used ($0.1–2.0 \times 10^8$ cells per flask).

Because of the brief contact which the leukocytes have with large amounts of citrate in the fibrinogen isolation method, several comparisons were made of results with this method and the phytohemagglutinin method in which heparin is the anticoagulant. Typical data, shown in Table 2, indicate no differences due to isolation technic.

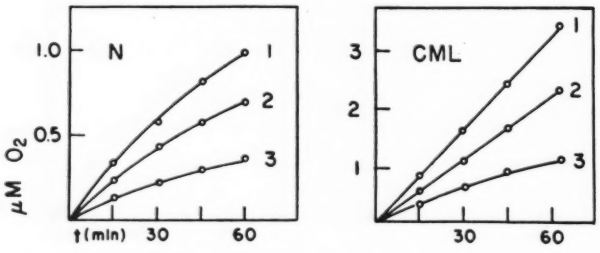

CHART 1.—Effect of varying amounts of homogenate on O_2 consumption. Measurements made using "complete" system. *N* = normal leukocytes; *CML* = chronic myelocytic leukemia leukocytes. Curve 1, 0.6 ml. homogenate; curve 2, 0.4 ml.; curve 3, 0.2 ml.

TABLE 2

EFFECT OF ISOLATION TECHNIC ON O_2 CONSUMPTION, GLUCOSE UTILIZATION, AND LACTIC ACID PRODUCTION*

(Chronic Lymphatic Leukemic Leukocyte Homogenate)

	Fibrinogen method			Phytohemagglutinin method		
System	O_2 cons.	Gluc. util.	Lactic prod.	O_2 cons.	Gluc. util.	Lactic prod.
"Complete"	0.06	0.29	0.44	0.06	0.30	0.43
"Complete" minus glucose and HDP	0.05	†	0.08	0.05	†	0.07

* Results are expressed in mM/10^{10} cells/hr.

† No glucose added.

RESULTS

Data on O_2 consumption, glucose utilization, and lactic acid production are presented in Table 3, which shows means of all results and standard deviations with P values.

It is seen that the O_2 consumption, glucose utilization, and lactic acid production of normal leukocyte homogenate in a system containing added ATP, DPN, and cytochrome c are significantly higher than in CML and CLL homogenates. Lactic acid production was significantly higher in CML than in CLL. The wide standard deviations in the N results may be due, in part, to the smaller number of cells used in some experiments with consequent magnification of error in cell enumeration, sampling, etc. In all experiments an effort was made to adjust the number of cells per flask to about 5×10^7, but because of the disparity in peripheral leukocyte count between normal and leukemic blood it was sometimes difficult to achieve this goal. Data given below show that computed metabolic rates per 10^{10} cells were not materially affected by cell concentrations.

Consistent results were not obtained in repeat determinations on the same individual, and radiation had no discernible effect.

DISCUSSION

The reported results emphasize the futility of labeling these cells as malignant, normal, or whatever on the basis of such metabolic performances in an arbitrarily devised system. It is true that

TABLE 3

COMPARISON OF OXYGEN CONSUMPTION, GLUCOSE UTILIZATION, AND LACTIC ACID PRODUCTION IN LEUKOCYTE HOMOGENATES*

	Type of leukocyte†			Tests of significance (P values)		
	N	CML	CLL	N vs. CML	N vs. CLL	CML vs. CLL
Oxygen consumption	0.40±0.17	0.12±0.03	0.11±0.01	<0.001	<0.001	>0.50
No. of exps.	39	23	10			
Glucose utilization	1.40±0.78	0.52±0.26	0.31±0.08	<0.001	<0.001	0.05
No. of exps.	12	9	7			
Lactic acid production	3.01±1.60	1.27±0.50	0.48±0.19	0.01	<0.001	<0.01
No. of exps.	10	9	7			

* Results are expressed in mM/10^{10} cells/hr. Values given are means of all determinations ± standard deviations.

† Abbreviations used: N = normal; CML = chronic myelocytic leukemic; CLL = chronic lymphatic leukemic.

leukemic leukocytes have high aerobic glycolytic rates, but this is equally true of normal leukocytes, so that these cells belong in the category of "unusual" tissues along with retina and kidney medulla. Using the data in Table 3, the ratio lactic acid production/O_2 consumption (expressing both activities in terms of glucose equivalents[3]) is found to be 29.2 for N, 31.0 for CML, and 14.6 for CLL. The ratios of N and CML are similar, both being about twice that of CLL. It should be noted that these results were obtained in an incubation mixture containing glucose and HDP as substrates.

The tissue reference unit for expression of data has traditionally been dry weight. This technic, satisfactory for tissue slices, has no advantages in the study of leukocytes over the more easily derived and certainly more plausible method of expressing results on a unit cell basis. The disadvantages of dry weights have been discussed by Bach (1). It is recognized that the unit cell basis is not necessarily a simplification, since the active centers in leukemic cells may be normal in mass and abnormal in activity rate, abnormal in mass and normal in rate, or abnormal in both mass and rate. A cellular basis for data would not discriminate between these three possibilities. If the parameters studied here are a function of the amount of cytoplasm, we may be dealing merely with a smaller amount of normally active substance. The same is true if the activity is located in the mitochondria, which may be low in number. This seems to be the case in mouse hepatoma, according to Schneider and Hogeboom (17). However, although mitochondrial nitrogen was low in their material, succinoxidase and cytochrome oxidase were even lower. There are no data on this point in leukocyte material. Studies are in progress in this laboratory on cell phosphorus, pentosenucleic acid, and desoxypentosenucleic acid as possible units of reference for metabolic data.

The data in Table 3 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O_2 consumption, it is seen that the amount of glucose utilized *exceeds* the amount of glucose "cleavage products" by 40 per cent in N, 18 per cent in CML, and 72 per cent in CLL. If the assumption is made that, *in this respect*, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = 0.18 and L = 0.72 and the normal differential is 65 per cent M and 35 per cent L, then

$$0.65 \times 0.18 + 0.35 \times 0.72 = 0.37,$$

a figure reasonably close to the observed 0.40 for normal leukocytes.

[3] One mM glucose is equivalent to 2 mM lactic acid and 6 mM oxygen.

SUMMARY

A detailed investigation of the dynamic biochemistry of leukocytes is an approach to the study of leukemia of possible importance. Leukocytes offer striking opportunities for the study of human material on the level of cellular biochemistry.

Data have been presented on the oxygen consumption, glucose utilization, and lactic acid production of homogenates of leukocytes isolated from normal (N), chronic myelocytic (CML), and chronic lymphatic leukemic (CLL) blood. Glucose utilization was determined by the highly specific glucose oxidase method which gives higher disappearance rates than the reducing method. Tissues were incubated in buffered media containing added DPN, ATP, cytochrome c, phosphate, Mg^{++}, and various substrates. Data were expressed per 10^{10} cells.

With glucose and hexose diphosphate as substrates, oxygen consumption, glucose utilization, and lactic acid production were significantly higher in N than in CML and CLL homogenates. Lactic acid production and glucose utilization were higher in CML than in CLL.

Leukocytes have a predominantly aerobic glycolytic metabolism. The ratio of aerobic glycolysis to respiration (in terms of glucose equivalents) is about 30 in N and CML and about 15 in CLL.

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The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia

II. The Effect of Various Substrates and Coenzymes on Glycolysis and Respiration*

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In the previous paper (1), data were presented on the aerobic carbohydrate metabolism of homogenates of leukocytes isolated from normal (N), chronic myelocytic leukemic (CML), and chronic lymphatic leukemic (CLL) bloods. It was shown that with glucose and hexose diphosphate (HDP) as substrates, oxygen consumption, glucose utilization, and lactic acid production were significantly higher in N than in CML and CLL homogenates.

There have been few reports on the effect of varying substrate and coenzyme conditions in leukocyte material. Marinelarena (5) reported the respiratory and glycolytic activities of guinea pig exudate leukocytes with various substrates and inhibitors. Using intact, washed cells suspended in Ringer's solution and conventional manometric technic, he typically found an O_2 consumption around 0.46 μl/10^6 cells/hr and glycolytic rate of 0.89 μl CO_2/10^6 cells/hr. Homologous serum benefited respiration. Added glucose, hexose diphosphate, and fructose increased glycolysis; succinate increased respiration. Glycolysis was decreased by fluoride and iodoacetate. KCN and malonate decreased respiration. Reports of various authors have shown that leukocyte respiration is decreased by saponin (7), potassium arsenite (12), and thiouracil (11) and is stimulated by menadione (10) and ascorbic acid (6).

This paper will report the effects on O_2 consumption, glucose utilization, and lactic acid production of diversification of added substrates and cofactors. The results show additional biochemical differences between normal and leukemic leukocytes and further support the view that classification of tissues on the basis of *in vitro* metabolic rates requires qualifying statements concerning the incubation medium employed.

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MATERIALS AND METHODS

Materials and methods have been described in detail elsewhere (1). The basic incubation system, referred to below as the "complete" system, was as follows (final concentrations in parentheses): KH_2PO_4–K_2HPO_4 buffer, pH 7.4 (0.01 M), ATP-Na[1] (0.0011 M), DPN[2] (0.0007 M), cytochrome c[3] (4×10^{-8} M), glucose (0.0053 M), HDP (0.0053 M), $MgCl_2$ (0.0053 M), and H_2O q.s. 2.8 ml. Tissue aliquot was usually 0.55 ml.

Pyruvate[4] and succinate[4] were used as substrates in several experiments. Pyruvate was made by dilution and neutralization of redistilled pyruvic acid. Solutions of sodium succinate were made up directly. Both were kept in 0.01 M stock solutions. 0.3-Ml. additions to incubations contained 20 μM and gave final concentrations of 0.011 M.

RESULTS

Effect on different substrates.—Typical experiments showing the effects of different substrate conditions on oxygen consumption, glucose utilization, and lactic acid production in N, CML, and CLL leukocyte homogenates are shown in Table 1.

If the activity of the "complete" system (containing as substrates 15 μM each of glucose and HDP) is assigned a comparative value of 100, the effects of different substrates can be expressed as percentages of the "complete" system. Charts 1, 2, and 3 show these results graphically, indicating the several substrate combinations and the comparisons with the glucose–HDP system. Results shown are the means of four to six experiments.

[1] Purchased from Pabst Laboratories, Milwaukee, Wis.

[2] Purchased from Schwarz Laboratories, New York, N.Y.

[3] Purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

[4] Purchased from Eastman Organic Chemicals, Inc.

On a percentage basis, variations in substrates affected *O_2 consumption* of N homogenates more profoundly than of CML and CLL material. The differences shown in Chart 1 between N and CML and N and CLL are statistically significant in the incubations containing succinate (P = 0.01–0.001). Differences between CML and CLL in the succinate-containing systems are probably not significant (P = 0.1–0.2), except in the pyruvate+succinate system where the CML vs. CLL P value is 0.001. In the presence of glucose, HDP, or pyruvate or all three, respiration remained unchanged. The decrease in O_2 uptake on omission of glucose and HDP was especially pronounced in the N material. The striking finding is the relatively greater respiratory stimulation of succinate in the normal and the insensitivity of leukemic homogenate respiration to substrate variation.

Percentile variation of *lactic acid production* under varying substrate conditions was similar in N, CML, and CLL, all differences among the three being statistically insignificant. Endogenous lactic acid production in the absence of added substrate is low in all three tissues, indicating that "*aerobic glycolytic rates*" *depend strictly on availability of substrate*. The presence of glucose or HDP alone yields almost as much lactic acid as both together. These points are illustrated in Table 2, where calculations are shown of the ratio lactic acid production/O_2 consumption (both activities expressed in terms of glucose equivalents)[5] with glucose and HDP present and with no added substrates present. It is seen that the preponderance of aerobic glycolysis over respiration is directly related to the availability of glycolyzable substrate. Note (*a*) the similarity of the ratios of N and CML in the presence of substrate, both being twice CLL and (*b*) the disparity of the ratios in the absence of added substrate, that of CML being higher than N and CLL.

When the substrate is pyruvate (in excess) and with DPN present, little lactic acid is formed unless glucose is added, probably indicating the need for some reaction sequence which will convert DPN to $DPNH_2$, the necessary coenzyme for the transformation of pyruvic to lactic acid. This point is receiving further study.

Glucose disappearance diminished in the absence of HDP, indicating the probable role of HDP as a primer of phosphorylation. The percentile effect of HDP omission was about the same in normal and leukemic homogenates. Even with HDP present, added succinate decreased the disappearance of glucose, suggesting some connection between increased oxidation due to succinate and the initial phosphorylation of glucose, possibly via the adenylic system. We cannot explain why glucose utilization is lower when succinate alone is added to glucose+HDP than it is when succinate and pyruvate are added.

Effect of variation in added ATP, DPN, and cytochrome c.—Comparisons were made of metabolic activity in the absence of added ATP, DPN, and cytochrome c and in the presence of these substances, alone and in various combinations. Reduced DPN[6] ($DPNH_2$) was added in certain experiments. Typical results for N, CML, and

TABLE 1

O_2 CONSUMPTION, GLUCOSE UTILIZATION, AND LACTIC PRODUCTION IN N, CML, CLL HOMOGENATES WITH VARIOUS SUBSTRATES

Typical experiments. All incubations contained homogenate, 0.55 ml.; phosphate buffer, pH 7.4, 0.15 ml.; ATP-Na, 3 μM; DPN, 2 μM; cytochrome c, 0.04 μM; $MgCl_2$, 15 μM; and water q.s. 2.8 ml. Amounts of substrate added: glucose and HDP, 15 μM; pyruvate and succinate, 30 μM. Boldface indicates "complete" system. Results are expressed in mM/10^{10} cells/hour.

	N			CML			CLL		
ADDITIONS	O_2 cons.	Gluc. util.	Lactic prod.	O_2 cons.	Gluc. util.	Lactic prod.	O_2 cons.	Gluc. util.	Lactic prod.
None	0.22	*	0.31	0.08	*	0.44	0.11	*	0.02
Gluc+HDP	**0.47**	**2.28**	**3.63**	**0.12**	**0.62**	**1.43**	**0.14**	**0.28**	**0.47**
Gluc	0.47	1.79	3.58	0.12	0.48	1.07	0.14	0.22	0.32
HDP	0.44	*	3.63	0.12	*	1.33	0.14	*	0.33
Succinate	0.88	*	0.27	0.15	*	0.23	0.17	*	0.03
Gluc+HDP+Succ	0.87	2.13	3.61	0.15	0.59	1.40	0.17	0.26	0.45
Gluc+Succ	0.89	1.65	3.49	0.15	0.60	1.20	0.17	0.22	0.33
HDP+Succ	0.88	*	3.52	0.16	*	1.34	0.17	*	0.34
Pyruvate	0.46	*	0.80	0.12	*	0.52	0.14	*	0.22
Py+Succ	0.94	*	0.58	0.18	*	0.41	0.18	*	0.18
Gluc+HDP+Py	0.47	2.25	3.61	0.12	0.62	1.51	0.14	0.27	0.45
Gluc+HDP+Py+Succ				0.12	0.64	1.51			

* No glucose added.

[5] One mM glucose is equivalent to 2mM lactic acid and 6 mM of oxygen.

[6] Purchased from Sigma Chemical Co., St. Louis, Mo.

CLL homogenates (with glucose and HDP as substrates) are shown in Table 3.

If the activity in the "complete" system (containing 3 μM at ATP, 0.04 μM of cytochrome c, and 2 μM of DPN) is assigned a relative activity of 100 per cent, percentile effects of various deletions and additions can be shown graphically (Chart 4). Results shown are the means of four to six experiments.

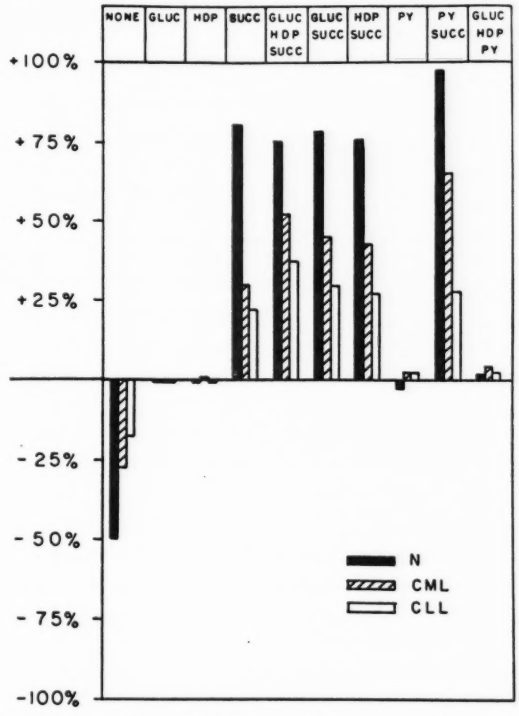

CHART 1.—Effect of different substrates on O_2 consumptions of *N*, *CML*, and *CLL* leukocyte homogenates. Activity in glucose and HDP ("complete" system) is arbitrarily assigned a comparative value of 100 per cent and activities with other substrates, alone and in combination, are plotted as a per cent difference from this activity. Incubation conditions are given in the legend of Table 1. Bar graphs show means of four to six experiments. All activities were calculated in terms of 10^{10} cells per hour.

It is seen that metabolic activity is markedly affected by the presence or absence of these particular cofactors. *Oxygen uptake* is decreased by omission of all three cofactors, the percentile effect on N being greater than on CML and CLL. The omission of DPN only or the replacement of DPN by reduced DPN causes a depression in oxygen consumption similar in degree to that following omission of DPN, ATP, and cytochrome c, in each case the N effect being greater. This would indicate that the homogenates require DPN for maximal oxygen consumption and are unable to derive it by oxidation of $DPNH_2$. ATP omission (but with DPN and cytochrome c present) causes a small decrease in O_2 uptake, again more striking with N homogenates. Omission of cytochrome c causes a greater lowering of O_2 consumption in the leukemic material than in the normal, producing, in fact, in CML and CLL a greater decrease than when cytochrome c, ATP, and DPN were all omitted. These results suggest that, when glycolysis is brisk, the depression of respiration due to

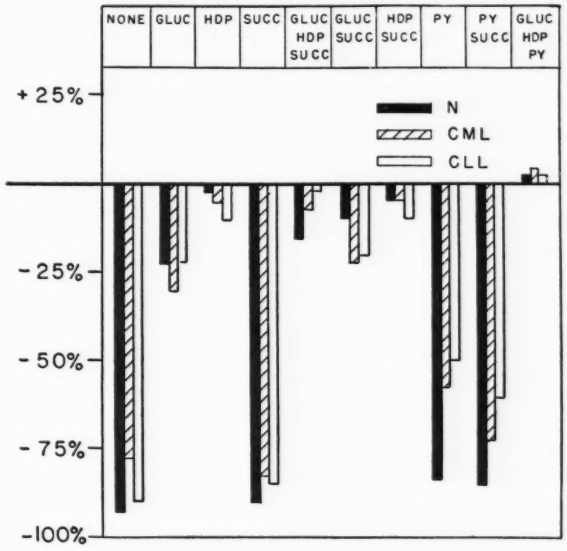

CHART 2.—Effect of different substrates on lactic acid production of *N*, *CML*, and *CLL* leukocyte homogenates. Explanation given in caption for Chart 1.

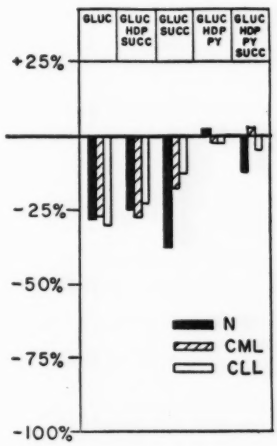

CHART 3.—Effect of different substrates on glucose utilization of *N*, *CML*, and *CLL* leukocyte homogenates. Explanation given in caption for Chart 1.

TABLE 2

RATIOS OF LACTIC ACID PRODUCTION/O_2 CONSUMPTION IN THE PRESENCE AND ABSENCE OF ADDED GLUCOSE AND HDP

Ratios computed in terms of glucose equivalents

Additions	N	CML	CLL
Glucose+HDP	29.2	31.0	14.6
None	3.5	10.0	1.6

cytochrome c deficiency is relatively greater than if glycolysis is sluggish. $DPNH_2$ seems actually to inhibit O_2 consumption in N homogenates, since activity was 100 per cent with 1 μM of DPN present but was 23 per cent lower with 1 μM each of DPN and $DPNH_2$ present, a significant difference. This did not occur with CML and CLL. The results show that O_2 uptake was the same in N, CML, and CLL with 1 μM of DPN as with 2 μM.

Glucose utilization requires added DPN and ATP, the requirement for DPN being proportionately the same in N, CML, and CLL and that for ATP being greater in N than in CML and CLL. Table 3 shows that if 6 μM at ATP is added instead of 3 μM, glucose utilization is less than maximal (in CML). Omission of cytochrome c had little effect on glucose utilization. Substitution of $DPNH_2$ for DPN lowered glucose utilization, simulating the effect of DPN omission. One μM of DPN with or without 1 μM of $DPNH_2$ was as effective in supporting glucose utilization as was 2 μM of DPN.

The diminution in *lactic acid production* after omission of ATP, DPN, and cytochrome c was greater in CML and CLL than in N. Interestingly, when all three of these cofactors are omitted, metabolic depression of N homogenate is reflected chiefly in O_2 and glucose uptake and least in lactic acid production, whereas leukemic material shows its greatest lability in lactic acid production and least in glucose utilization and oxygen consumption. DPN omission causes a large decrease in lactic acid production in CML and CLL but only a slight decrease in N. ATP and cytochrome c omission cause only slight decreases in lactic acid production. CLL reacts differently than CML in its response to DPN/$DPNH_2$ manipulation. Substitution of $DPNH_2$ for DPN lowers lactic acid production in CML and CLL (to a greater extent than in N) but addition of DPN (1 μM) or DPN (1 μM)+$DPNH_2$ (1 μM) depresses only CLL and not CML or N. One μM of DPN adequately supports O_2 uptake and glucose utilization in CLL but appears to be inadequate for maximal lactic acid production. This could pos-

TABLE 3

EFFECT OF VARIATION IN ADDED COENZYMES ON O_2 CONSUMPTION, GLUCOSE UTILIZATION, AND LACTIC ACID PRODUCTION IN N, CML, AND CLL LEUKOCYTE HOMOGENATES

Typical experiments. All incubations contained homogenate, 0.55 ml.; phosphate buffer, pH 7.4, 0.15 ml.; glucose, 15 μM, HDP, 15 μM; $MgCl_2$, 15 μM plus additions. Boldface indicates "complete" system.
Results are expressed in mM/10^{10} cells/hour.

Additions (in μM)				N			CML			CLL		
DPN	$DPNH_2$	ATP	Cyto. c	O_2 cons.	Gluc. util.	Lactic prod.	O_2 cons.	Gluc. util.	Lactic prod.	O_2 cons.	Gluc. util.	Lactic prod.
0	0	0	0	0.11	0.56	1.68	0.07	0.29	0.76	0.07	0.34	0.42
2	0	0	0.04	0.26	0.62	1.70	0.10	0.30	1.33	0.08	0.38	0.67
0	0	3	0.04	0.18	1.00	1.92	0.08	0.30	0.88			
2	0	3	0	0.30	1.45	2.12	0.05	0.44	1.42	0.05	0.46	0.80
2	**0**	**3**	**0.04**	**0.35**	**1.47**	**2.15**	**0.11**	**0.46**	**1.51**	**0.09**	**0.48**	**0.87**
2	0	6	0.04				0.11	0.39	1.51			
1	1	3	0.04	0.27	1.46	2.00	0.11	0.42	1.52	0.09	0.48	0.60
0	2	3	0.04	0.13	1.34	1.95	0.07	0.35	0.98	0.06	0.40	0.48
1	0	3	0.04	0.35	1.46	2.00	0.11	0.48	1.31			

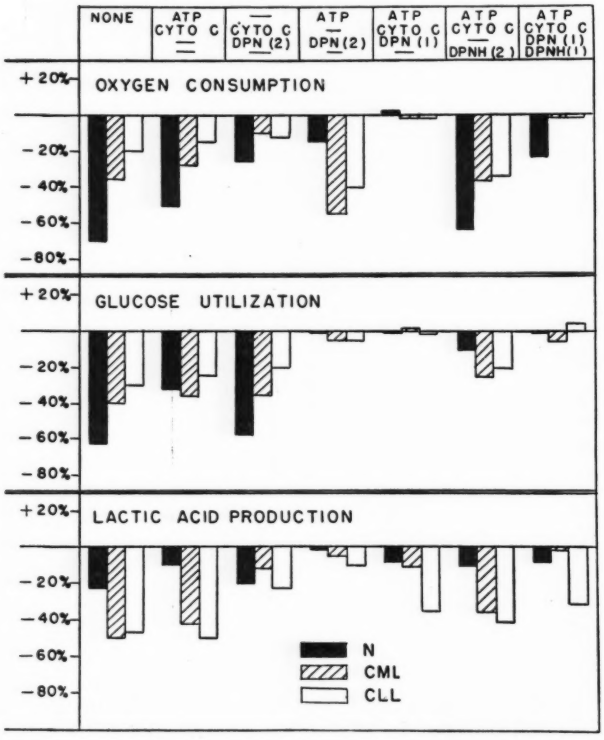

CHART 4.—Effect of variation in added ATP, DPN, and cytochrome c on O_2 consumption, glucose utilization, and lactic acid production in *N*, *CML*, and *CLL* leukocyte homogenates. Arbitrary activity of 100 per cent is assigned to system containing ATP 3 μM; cytochrome c, 0.04 μM; and DPN, 2 μM. Graph shows comparative activity of systems containing only additions indicated. DPN (1) and DPNH (1) refer to addition of only 1 μM of these substances. Incubation conditions given in the legend for Table 3. Bar graphs show means of four to six experiments. All activities calculated in terms of 10^{10} cells per hour.

sibly be related to the above-described differences in the ratios of glycolysis to respiration between CLL and both CML and N, CLL showing a smaller ratio.

These results illustrate (*a*) the need for added ATP, DPN, and cytochrome c for maximal activity in all three tissues; (*b*) the striking differences in response to various deletions and additions between normal and leukemic material, the normal showing greater lability to environmental change in its O_2 and glucose consumption and the leukemic in lactic acid production; (*c*) the greater cytochrome c requirement of leukemic homogenates for maximal oxygen uptake and the greater DPN requirement of CLL homogenates for maximal lactic acid production.

DISCUSSION

The reported results indicate certain qualitative differences in metabolic behavior between normal and leukemic leukocytes. Glycolysis/respiration ratios vary significantly in the presence and absence of added substrate. CLL material shows a higher ratio of respiration to glycolysis than CML and N, although, in the presence of glucose, it still has a predominantly aerobic glycolytic metabolism. In leukemic cells, there seems to be a greater indifference to substrate, at least in the oxidative cycle, and, conversely, a markedly greater response to addition or deletion of cofactors, particularly DPN and cytochrome c. The relative effects of substrate withdrawal and succinate stimulation are notably smaller in leukemic material.

Roskelley, Mayer, Horwitt, and Salter (8), Greenstein (3), and others found with many normal tissues (but not all) much greater respiratory stimulation on adding succinate than occurred with tumor tissue, suggesting some deficiency in the tumor succinoxidase system—possibly in the effective cytochrome c level. Addition of succinate *and cytochrome c* caused a much greater percentile increase in the O_2 consumption over that with succinate alone in malignant tissue than in normal tissue. This suggested to the authors either a deficiency of cytochrome c in tumor or some shift in the redox equilibrium so that a greater amount of the cytochrome c present is in a reduced state in tumors than in normal tissue. Actual analyses of various tissues by DuBois and Potter (2) showed decidedly lower cytochrome c levels in tumors than in most normal tissues. Shack (9) found that O_2 uptake of hepatoma slices is limited by the cytochrome system whereas normal liver and other normal tissues had a cytochrome reserve. The results presented in Table 3 show that with glucose and HDP as substrates, omission of cytochrome c causes a greater percentile decrease in O_2 consumption in CML than in N.

The observed DPN dependence recalls a study of Wenner, Spirtes, and Weinhouse (13) in which suspensions of tumor mitochondria were found capable of a "normal" rate of pyruvate oxidation if, in addition to M^{++}, ATP, and cytochrome c, an adequate amount of DPN was added. Further studies on the specific role of DPN and ATP in the metabolism of leukemic leukocytes are now in progress in our laboratory.

Of interest is the reduction in lactic acid production in CLL when only 1 μM of DPN is added, and striking differences in lactic acid production between N, CML, and CLL on manipulation of added DPN and $DPNH_2$. In an effort to clarify these results, measurements are being made of endogenous levels of DPN and $DPNH_2$, and an attempt is being made to demonstrate a DPNase which could influence the apparent DPN requirement as has been shown in brain tissue (4).

SUMMARY

Data have been presented on the oxygen consumption, glucose utilization, and lactic acid production of homogenates of leukocytes isolated from normal (N), chronic myelocytic (CML), and chronic lymphatic leukemic (CLL) blood. Studies were made of the effect of the following substrates in varying combinations: glucose, hexose diphosphate, pyruvate, and succinate. Effects of varying added DPN, $DPNH_2$, ATP, and cytochrome c were also studied. Data were expressed per 10^{10} cells.

Although leukocytes have a predominantly aerobic glycolytic metabolism, the relative preponderance of aerobic glycolysis over respiration depends strictly on the availability of glycolyzable substrates. The glycolysis to respiration ratio is much higher when glucose is present than when it is absent. Of the three tissues, CLL homogenates have the lowest ratio of glycolysis to respiration.

Qualitative as well as quantitative differences in metabolic performance occur on manipulation of substrates. Succinate stimulates oxygen uptake to a greater extent in N than in leukemic material. With pyruvate as the substrate, and with DPN present, lactic acid production is low unless glucose is added.

Omission or manipulation of added cofactors profoundly affected metabolic rates. N, CML, and CLL homogenates required added ATP, DPN, and cytochrome c for maximal activity. Leukemic leukocytes had a greater cytochrome c requirement for maximal oxygen uptake than N cells.

CLL homogenates had a greater than normal DPN requirement for maximal lactic acid production. Normal cells showed a greater lability to cofactor manipulation in their oxygen and glucose consumption, while leukemic cells were most variable in their lactic acid production.

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The Effect of Oxygen Concentration upon the Induction by X-Rays of Melanotic Tumors in *Drosophila melanogaster*

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Because of the vast background of genetic knowledge of *Drosophila melanogaster* and because of the relative ease with which environmental conditions affecting the fruit fly may be controlled, to a degree much more difficult to establish and maintain for the higher animals usually used in cancer research, studies of the environmental and genetic factors that affect tumor production in Drosophila have been undertaken by a considerable number of workers. The earlier investigations dealt mainly with the genetic differences, but more recent studies have extended to the effects of temperature (9, 10), crowding and feeding (1, 3, 11, 12, 14, 18) and x-rays (2, 8). A great portion of this work has been summarized by Scharrer and Lochhead (15).

METHODS

The present study deals with the effects of different oxygen concentrations at the time of irradiation upon the induction of tumors by x-rays. Preliminary studies by the authors (7) showed a great difference between the frequencies of tumors produced in an Oregon-R inbred strain and in our isogenic suppressor-erupt stock when either is exposed in the embryonic period to an x-ray dose of 1,000 r (60 kv., 15 ma., 12 inches distance, 80–100 r.p.m.). The incidence of tumors in the x-rayed Oregon-R stock was only 10.1 per cent, but an incidence of 81.7 per cent was obtained in the x-rayed suppressor-erupt stock. The latter incidence is much higher than any of the values found by Hartung (8) following application of a similar dose to any one of the three tumorous strains he studied. In fact, it is considerably higher than the greatest incidence he was able to obtain at the highest dose tolerated by the fly embryos. Since the nonirradiated suppressor-erupt control flies manifest an incidence of only 4.4 per cent, it was felt that this stock would be ideal to use in further studies of the production of tumors. For comparison, the Oregon-R inbred strain, with a norma incidence of tumors of 1.0 per cent, was used.

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Eggs were collected over a 2-hour interval and were exposed to the treatments at an embryonic age of 10 ± 1 hr., as in previous work by the authors (7). An x-ray dose of 1,000 r was used in all cases, with the temperature kept at 25 ± 1° C. The eggs were treated in a lucite chamber filled with gas of a desired composition. Following treatment, the embryos were placed in 4-inch petri plates containing only moist filter paper; 300–400 eggs were placed in each dish. After they had hatched, the larvae were fed entirely on dried brewers' yeast, moistened with distilled water. Fresh yeast was added daily.

Tumorous strains raised in crowded cultures have been found to show a reduced incidence of tumors (18). Friedman *et al.* (3) clearly showed this to be due to a nutritional deficiency, and Burdette (1) and Herskowitz and Burdette (11) found that tumor incidence is always highest in well yeasted cultures. Many workers have therefore placed small numbers of larvae in separate cultures in order to insure uncrowded conditions and a liberal food supply. Our studies show that this factor may be overcome by supplying an abundant quantity of yeast and by adding more daily, so that there is always more food present than the larvae require. Another factor responsible for variable incidence of tumors is the species of yeast used for nutrition (12). Since we have used the same yeast throughout, variation of this sort could not affect our findings.

The tumors do not appear as pigmented bodies until late in the third larval instar, although they are morphologically discernible some time earlier. The tumors have been examined histologically both from freshly dissected larvae and in sectioned material. Like the tumors described by Russell (13), they consist of masses of cells which are heavily pigmented in some areas and which are free-floating in the hemocoel. In certain aspects they resemble the phagocytic cells of the blood-forming organs (16, 17), but they do not correspond at all to imaginal disc cells, which are considerably smaller.

The first appearance of the tumors as discrete black bodies is followed by their growth and increase in number. As described by Russell, the tumors cease to grow or to increase in number at the time of pupation, although they may be carried over into the adult as benign tumors. The growth and increase in number of the tumors during the larval period necessitate selection of a standard time for counting larvae and tumors so as to make all series comparable. Prior to pupation the larvae crawl up the sides and onto the cover of the petri plate. This occurred in our cultures on the fifth day after treatment, about 96 hours after hatching; but in the x-rayed series pupation itself was delayed as much as 20–25 hours, if it occurred at all. It therefore seemed best to examine

the larvae on the fifth day, after they had left the food. The larvae classified were removed and placed in vials containing moist filter paper and yeast. Each larva was classified as possessing zero, one, two, three, or more than three tumors, in the last class the number running up to eight or more.

RESULTS

The influence of O_2 concentration upon the incidence of tumors produced by x-rays.—The number of tumors found in the two stocks irradiated in the different oxygen concentrations is shown in Table 1. Each series was composed of at least two duplicate runs, which in all cases were found not to differ statistically in tumor incidence. It is clear from the data that as the oxygen concentration is increased there is (*a*) an increase in the incidence of tumors, and (*b*) an increase in the number of tumors per affected larva. These data are summarized in those columns of Table 1 which give the percentage of incidence of tumors and the average number of tumors observed per larva, respectively. The latter represents a minimum, since the class "greater than three" was assigned the minimum value of four, although actually the average number of tumors per larva in this class may have been nearer six. The percentage of tumor incidence at the different oxygen concentrations used is shown graphically in Chart 1. Up to the concentration of O_2 in air, the incidence is seen to increase in direct linear proportion to the increase of oxygen concentration. The increased incidence between 20 per cent O_2 (air) and pure oxygen, though significant, is not so striking. Until intermediate concentrations of O_2 have been tested, it cannot be said whether this represents a gradual asymptotic approach or whether the curve rises to a maximum at some intermediate point and then falls.

TABLE 1

THE PRODUCTION OF MELANOTIC TUMORS IN LARVAE OF TWO STRAINS OF *Drosophila melanogaster* BY IRRADIATION WITH X-RAYS AT VARIOUS CONCENTRATIONS OF OXYGEN

	No. of larvae examined	No. with tumors	Larvae with given no. of tumors					Total no. tumors	Av. tumors per larva
			0	1	2	3	>3		
A. Oregon-R stock									
Unirradiated (2 series)	400	0 (0.0)*	400 (100.0)	0	0	0	0	0	0.000
1,000 r in N_2 (3 series)	800	1 (0.1)	799 (99.9)	1	0	0	0	1	0.001
1,000 r in $\frac{10 \text{ per cent } O_2}{90 \text{ per cent } N_2}$ (2 series)	800	35 (4.38±0.72)	765 (95.6)	28	7	0	0	42	0.053
1,000 r in $\frac{20 \text{ per cent } O_2}{80 \text{ per cent } N_2}$ (2 series)	800	81 (10.13±1.06)	719 (89.9)	68	11	2	0	96	0.120
1,000 r in 100 per cent O_2 (2 series)	800	115 (14.38±1.24)	685 (85.6)	93	22	0	0	137	0.171
B. Suppressor-erupt stock									
Unirradiated (4 series)	1,800	79 (4.4±0.47)	1,721 (95.6)	71	7	0	1	89	0.049
1,000 r in N_2 (2 series)	800	455 (56.9±1.75)	345 (43.1)	404	42	7	2	517	0.646
1,000 r in $\frac{10 \text{ per cent } O_2}{90 \text{ per cent } N_2}$ (2 series)	800	555 (69.38±1.6)	245 (30.6)	363	117	44	31	853	1.066
1,000 r in $\frac{20 \text{ per cent } O_2}{80 \text{ per cent } N_2}$ (3 series)	1,400	1,144 (81.71±1.03)	256 (18.3)	898	176	43	27	1,487	1.062
1,000 r in 100 per cent O_2 (4 series)	1,500	1,451 (96.73±0.45)	49 (3.3)	632	470	209	140	2,763	1.842

* Figures in parentheses are percentages with their standard errors.

The increased incidence of tumors with higher O_2 concentration alone.—The significant differences found when the embryos are x-rayed in different concentrations of oxygen make it important to know what the effects might be of varying the oxygen concentration, without any other treatment. A comparison of the three types of control series, exposed respectively to N_2, air, and O_2, but not x-rayed, is given in Table 2. The exposures to N_2 or O_2 were for 10 minutes during the embryonic period at age 10 ± 1 hr., the age at which most of the series reported in Table 1 were x-rayed. It is clear that both the Oregon-R and the suppressor-erupt strains show a small but significantly in-

creased incidence of tumors with increasing O_2 concentration. These results make it apparent that the radiogenic effect consists of an enormous enhancement of the differential effects of oxygen tension alone.

It may also be noted that the Oregon-R flies in the series in Table 2 showed a higher incidence of tumors than in the control series reported in Table 1. This is an indication that in this strain there is probably some heterogeneity for the genetic factors leading to the development of tumors.

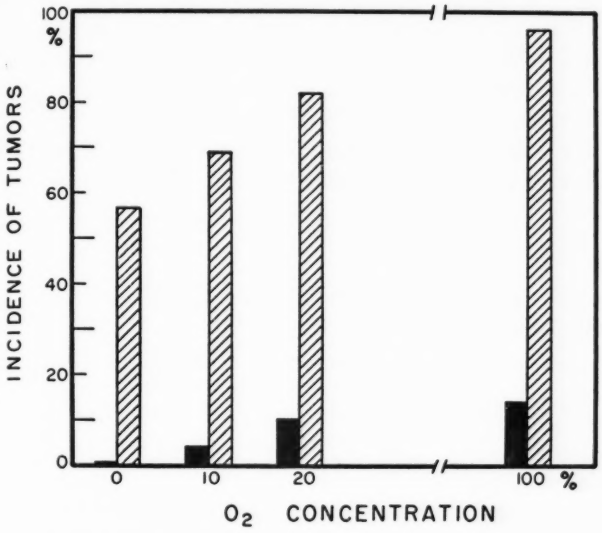

CHART 1.—The incidence of melanotic tumors in third instar larvae of *Drosophila melanogaster* x-rayed in various concentrations of oxygen. Solid bars, Oregon-R wild-type stock; shaded bars, suppressor-erupt isogenic stock.

The influence of O_2 concentration upon effects of x-rays on mortality and rate of development.—In addition to the killing effect of the x-rays that is manifested in the larval and pupal periods and that also increases with the oxygen concentration at the time of irradiation (7), the rate of development is affected, the amount of delay and prolongation of development also increasing with increased concentration of O_2 at the time of the x-ray treatment. Development through the second larval instar was normal for all embryos which hatched, but the third instar was prolonged, and pupation was considerably delayed for all series except that which was x-rayed in pure nitrogen. In the air and pure oxygen series many larvae, before pupating or dying, lived far beyond the time at which pupation occurred for others in the same series. In several cases in the pure oxygen series the larvae continued to live for 10 days extra, that is, for a total of 14 days without pupating. It is surprising to find such variations for larvae of the same age (± 1 hr.) and raised under identical conditions. Many more individuals died in the pupal stage. Yet those adults which succeeded in eclosing frequently contained tumors. Hence these growths are hardly accountable *per se* for the larval and pupal mortality.

In his study of temperature effects on the incidence of tumors, Hartung (9) found that his three tumorous strains consistently showed their highest incidence of tumors at the lowest temperature, at which of course the developmental rate was considerably slower (10). This was interpreted to mean that a slow developmental rate conduces to raising the penetrance of tumors, an effect superimposed on the somewhat different rates of development of the several tumor strains. On the other hand, Wilson (19) found that, upon prolonging the larval stage with 2,4-dinitrophenol, the appearance of the tumors was delayed although the incidence was not altered. Again, Burdette (1) and Herskowitz and Burdette (11) found that when pupation is delayed by impaired nutrition, the incidence of tumors decreases with degree of nutritional deficiency. Whether these contradictions are due to the use of different tumorous strains by these work-

TABLE 2

THE EFFECTS OF EXPOSING EMBRYOS OF TWO STRAINS OF *D. Melanogaster* TO VARIOUS CONCENTRATIONS OF OXYGEN

	Total	No. with tumors	Incidence ± S.E. (per cent)	Total no. tumors
Suppressor-erupt stock				
1. 0 per cent O_2 (N_2)	400	10	2.5±0.8	10
2. 20 per cent O_2 (Air)	400	19	4.8±1.1	23
3. 100 per cent O_2	400	24	6.5±1.2	35
$\chi^2=5.59$		D.F.=2	P=0.10–0.05	
$t_{1,3}=2.66$		P<0.01		
Oregon-R stock				
1. 0 per cent O_2 (N_2)	400	3	0.75±0.25	3
2. 20 per cent O_2 (Air)	400	8	2.0±0.7	10
3. 100 per cent O_2	400	19	4.7±1.06	23
$\chi^2=13.75$		D.F.=2	P=0.001	

ers, or whether the prolongation of development is incidental to other effects of temperature, nutrition, or chemicals on the growth of tumors, remains uncertain.

In the present studies, a higher O_2 concentration at the time of treatment not only increases the incidence of tumors but also increases the length of larval life, and hence permits a more accurate evaluation of the relationship between the two. This was carried out in the following way. From the same suppressor-erupt cultures of eggs laid within a 2-hour period and from which treated larvae selected at random had been counted on the fifth day, other larvae were examined on the sixth day, exactly 24 hours later. All series were replicated, and duplicate series were never found to differ significantly. On the other hand, the incidence of tumors on the fifth and sixth days, shown in Table 3, differed significantly in every case. It is evident that the number of unaffected larvae decreased, while the number of tumors per larva increased in each of the series. The incidence of tumors increased by 10–17 per cent over the 24-hour period. Thus, in those larvae whose development was prolonged by 24 hours there was a significant increase in both incidence of tumors and number per larva. It is further apparent from the data in Table 3 that the larval-tumor comparisons for the different concentrations of O_2 could have been made on the sixth day just as well as on the fifth. If the difference in developmental rate of the series treated in nitrogen, which developed somewhat more rapidly than the others, is taken into account, so that the fifth day incidence for the N_2 series is compared to the sixth day incidence for the other series, the differences found become even more striking.

TABLE 3

THE INCREASE IN NUMBER OF TUMORS WITH ADVANCING LARVAL AGE IN THE SUPPRESSOR-ERUPT STOCK OF *Drosophila melanogaster*

	Day of class.	Total no.	Larvae with given no. of tumors					Av. tumors per larva
			0	1	2	3	>3	
1,000 r in N_2	5th	600	267 (44.5)*	309	18	5	1	0.607
	6th	400	110 (27.5)	157	82	29	22	1.240
1,000 r in $\frac{10 \text{ per cent } O_2}{90 \text{ per cent } N_2}$	5th	400	131 (32.7)	183	52	21	13	1.005
	6th	400	65 (16.3)	179	92	34	30	1.463
1,000 r in $\frac{20 \text{ per cent } O_2}{80 \text{ per cent } N_2}$	5th	400	73 (18.3)	196	94	23	14	1.273
	6th	400	34 (8.5)	168	119	45	34	1.693

* Figures in parentheses are percentages.

DISCUSSION

The effect of the x-rays upon the suppressor-erupt gene present in the stock used in these studies, and also in many other laboratory stocks and captured populations, is to block the action of this specific gene and thus to allow the frequently present but suppressed mutant gene erupt (*er*) to manifest itself (4–7, and unpublished results). This mutant produces a particular type of abnormal growth, taking the form of an eruption of hypodermal tissue through the eye, nearly always in its very center. In extreme cases the growth may bear small bristles and appear to be segmented, in such instances resembling a palp or abortive antenna. Studies of the effect of the oxygen concentration prevailing at the time of irradiation with x-rays show that, with increasing O_2 concentration, there is an increasing inhibition of the action of the suppressor gene and consequently an increasing expression of erupt (7). The frequency of manifestation of erupt in flies that were x-rayed in air is somewhat higher than the incidence of tumors, and the incidence of erupt diminishes much more rapidly with decreasing oxygen tension than the decrease in the incidence of tumors. This does not alter the general similarity of the dependence of both phenomena upon the oxygen concentration at the time of irradiation, and may mean simply a difference in the thresholds of the two effects. What seems most significant is (*a*) that both phenomena show a direct linear proportionality to the oxygen concentration at the time of irradiation and (*b*) that both are clearly a function of the genetic constitution of the strain studied.

A current theory regarding the induction of malignant growths by ionizing radiations such as x-rays is that the radiation produces its effect by destroying some substance which normally acts as a suppressor of uncontrolled growth, a substance that furthermore is under genetic control. The present case involves all the elements needed to investigate and test such a theory. Moreover, this

genetic system seems highly suitable for further studies of (*a*) the nature of the induction of uncontrolled growth by ionizing radiations and its dependence upon the presence of oxygen, (*b*) the nature and mode of action of substances that protect against radiation injury, and (*c*) the relation of hereditary factors to all these.

SUMMARY

1. Studies of a particular stock of *Drosophila melanogaster* have shown that it contains a suppressor gene that inhibits the manifestation of erupt, a mutant that produces an abnormal growth of hypodermal tissue which erupts through the center of the eye. The action of the suppressor-erupt gene is blocked when the embryos are x-rayed.

2. The same stock possesses an unprecedented tendency to produce melanotic tumors when the embryos are x-rayed.

3. Both the incidence of tumors and the incidence of erupt increase significantly and linearly for increasing O_2 concentrations at the time of irradiation, over the range from 0 to 20 per cent. Although there is only a small further increase as the shift is made from air to pure oxygen, this increase is also significant for both effects.

4. In addition, both the mortality and duration of development also increase with increasing oxygen concentration at the time of x-ray treatment.

5. The same differential responses to x-rays in varying concentrations of oxygen also occur in another tested stock, Oregon-R, which is not closely related to the suppressor-erupt stock; but in the case of the Oregon-R stock all responses show a much lower incidence. Thus, it seems possible that the system affected is a general one, at least for *Drosophila melanogaster*.

6. The incidence of melanotic tumors in both stocks is slightly, but significantly, increased by exposure of the embryos for 10 minutes to pure O_2, without x-ray treatment. It is decreased by similar exposure to pure N_2. In this respect the tumor response differs from the response of suppressor-erupt, which is not affected by oxygen alone, in the absence of x-ray treatment.

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Studies on the Walker Tumor*

I. Standardization of the Growth of a Transplantable Tumor

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In this paper a standardized reproducible technic for obtaining non-necrotic transplantable tumors of uniform size and growth rate will be described, and the principal factors contributing to variability in growth rate will be analyzed. A number of potential objections have been raised to the extrapolation of results obtained with transplantable tumors to tumors of spontaneous origin (e.g., extreme malignancy and anaplasia, rapid growth, extensive necrosis, and rarity of metastasis); nevertheless, transplantable tumors exhibit all the salient biological and biochemical characteristics of malignant tissue, and resemble these to a far greater extent than normal tissues. Moreover, they provide the only practical method of studying reproducible malignant growths under controllable conditions during a reasonably short time period.

The transplantable tumor selected for this study was the Walker carcinosarcoma 256. This tumor was discovered on the lower abdomen of a pregnant albino rat in 1928. It regressed during lactation but again began to grow rapidly after weaning. The early history of the tumor and its histology have been extensively reviewed by Earle (1). The initial histological appearance of this tumor was that of an adenocarcinoma (presumably arising from mammary tissue) with a large proportion of connective tissue elements. Organized epithelial elements are no longer evident, and now the histological appearance is that of a highly anaplastic growth (Fig. 1). The Walker rat tumor was selected for these studies because of its uniformly rapid growth rate, the rarity of early regression, and the almost universal susceptibility of a variety of strains of rats.

Quantitative experimentation with transplantable tumors presents a variety of difficulties. The rapid growth of these highly malignant neoplasms makes them subject to early liquefactive central necrosis which confounds accurate measurement of their size, since the weight of a necrotic tumor does not constitute a quantitative measure of the amount of neoplastic tissue formed. Tumor weight may be regarded meaningful only when necrosis is minimal in a tumor sufficiently large so that the errors intrinsic in the dissection are relatively small.

MATERIALS AND METHODS

Transplantation.—Tumor transplantation was performed aseptically. The tumors were obtained from albino rats of either sex bearing subcutaneous or intramuscular transplants usually 7–10 days old. The viable portions of the tumor were sliced into small pieces and separated from connective tissue and necrotic portions. Each volume of tumor was mixed with approximately an equal volume of 0.9 per cent sterile sodium chloride solution in a graduated tube, and 2,000 units of crystalline penicillin G per milliliter of mixture was added. This mixture was transferred to a sterile, loosely fitting glass homogenizer and a cell suspension obtained by grinding the glass parts gently together by hand. 0.2 Ml. of the tumor suspension could then be readily injected into any desired location by means of a tuberculin syringe fitted with a 20-gauge needle.

Animals.—All animals were albino rats of either sex derived from the Sprague-Dawley (Holtzman) strain, Madison, Wisconsin. They were either procured from commercial firms or bred in our own colonies. Animals were maintained in stainless steel cages in air-conditioned quarters at 74°–78° F. with *ca.* 40 per cent relative humidity. Wild rats[1] of assorted ages and sex were captured by the City of Chicago Health Department. All animals were weighed at the time of tumor implantation (initial weight) and periodically during the period of tumor growth. The final total weight was determined just prior to the dissection of the tumors, and the carcass weight calculated by subtracting the weight of the tumors from the total weight. The difference between the initial weight and

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[1] The authors are indebted to Dr. Herman N. Bundesen, Commissioner of Health, City of Chicago, for this material.

the final carcass weight was taken as an expression of the change in carcass weight.

Diet.—Stock animals were maintained on Derwood pellets, Derwood Mills, Inc., Derwood, Maryland, or Rockland Farms Rat Pellets, Arcady Farms Milling Company, Chicago, and ample drinking water was available at all times.

RESULTS

Selection of implantation site.—The Walker tumor grows with great facility in a variety of locations in the rat. Slower growth is obtained subcutaneously than in intramuscular or intraperitoneal sites. The incidence of true nontakes in our rats is about 4 per cent (see below), and we have defined this as absence of macroscopically detectable tumors, or those weighing less than 0.5 gm. at the end of 8–10 days of growth in control animals. Subcutaneous tumors fall into discontinuous categories of size, so that there appears to be a group of small tumors as well as those which are considerably larger. Walpole (3) has already pointed out this difficulty which, however, he attributes to differences in susceptibility to the tumor, and has circumvented this by selecting the larger half of the tumors for analysis (a manipulation of the data which is not free from objection). The principal factor contributing to the variability of the size of subcutaneously implanted tumors is whether the tumor grows down through the deep fasciae, becomes attached to the muscle and draws its blood supply therefrom, or whether it remains superficial and attached to the skin. Despite careful implantation technic it is difficult to control the site of the inoculation absolutely. Moreover, subcutaneous vascularization is inferior to that found in tumors growing in the muscle, so that subcutaneous tumors reach a conveniently measurable size later and appear for a given size to be more necrotic than intramuscular tumors. The site of subcutaneous implantation also influences the rate of growth. The loose space of the middle of the back supports larger tumors than the tightly constricted subcutaneous space at the base of the tail. After examining a variety of subcutaneous and intramuscular implantation sites, we have found that inoculations into the flexor muscles of the thighs provides the best locations for well vascularized, symmetrical, uniform tumor growth. The implantation is most readily accomplished with the animal held by one person with its ventral surface facing up and both legs extended, while a second person pushes the skin taut over the flexor muscles, locates the body of the muscle between thumb and forefinger of one hand, while introducing the needle point of the tuberculin syringe and injecting the tumor inoculum with the other hand. 0.2 Ml. of tumor suspension is injected into each site. No difficulty has been experienced in dissecting intramuscular tumors cleanly and obtaining them free from muscular or connective tissue coverings. At the end of 8–10 days, tumors have reached a size convenient for dissection. The animals are killed with ether, the tumors dissected and weighed immediately on an analytical balance.

Incidence of successful takes.—During the course of these studies, 782 rats of the Sprague-Dawley strain were inoculated with Walker tumor into both flexor muscles of the thighs. In these animals bilateral failure of tumor growth was observed in 3.8 per cent, whereas only one tumor grew in approximately 1 per cent of the animals (Table 1).

TABLE 1

INCIDENCE OF SUCCESSFUL TAKES OF BILATERAL INTRAMUSCULAR WALKER TUMOR IMPLANTS IN ALBINO RATS

Sex	No. rats	No. animals without tumors	No. animals with only one tumor
Male	200	5 (2.5 per cent)	1 (0.5 per cent)
Female	582	25 (4.3 per cent)	7 (1.2 per cent)
Total	782	30 (3.8 per cent)	8 (1.0 per cent)

No difference in tumor susceptibility was observed in males or females. Animals in which the tumor once failed to grow bilaterally were almost invariably resistant to repeated subsequent implantations.

Analysis of the tumor sizes in all animals appears to indicate a certain symmetry in the tumors on either side of the animal. There is statistically less size variation between tumors in one individual than between tumors of any two individuals. These observations indicate that the principal factors contributing to variation in susceptibility or tumor size are biological rather than technical. The implantation of two tumors into each animal serves as a check on the implantation technic.

Size of Inoculum.—Variations in size of the initial inoculum were investigated to determine to what extent this factor controlled the incidence of positive takes and the size of the resultant tumor. Bilateral intramuscular injections, 0.2 ml. each, of standard tumor cell suspensions and two serial dilutions were administered to groups of male albino rats (200–250 gm. body weight). A twofold dilution of injected tumor did not significantly change the average size of tumors obtained after 10 days, but a fourfold dilution began to affect the results obtained (Table 2). Thus, small, unavoidable errors in inoculation technic do not affect the magnitude of the tumor obtained or the propor-

tion of successful takes. It may be recalled that Schrek (2) found that while the inoculation of 0.1 ml. of a suspension of Walker tumor resulted in 96 per cent takes, a dose of 0.01 ml. produced tumors in only 54 per cent of the rats.

Comparison of growth in albino and wild rats.—Study of the growth of the Walker tumor in a few wild rats showed that the tumor took well but grew at a somewhat slower rate. The rate of growth in wild rats is compared to that in female albino rats in Table 3.

Sex and size of animals.—A quantitative evaluation of the effect of sex and size of the host on the growth rate of the Walker tumor was undertaken. Male rats weighing initially an average of 356 gm. (4 months old) and female rats with an initial weight of 278 gm. (6 months old) were inoculated with bilateral intramuscular tumors, and groups of six rats were subjected to daily serial sacrifice from day 5 to day 10 of tumor growth. The growth rates, as shown in Chart 1, are essentially identical. On day 10, the male rats had gained weight to a total of 372 gm. (carcass weight = 343 gm.), whereas the females had lost slightly, weighing 272 gm. (carcass weight = 242 gm.); the final single tumor weights in the two groups were 14.3 gm. in the females and 14.7 gm. in the males.

Three narrow-range weight groups of male rats (ten animals each) were selected (between 169 and 454 gm.) and the size of bilateral intramuscular tumors on the eighth day determined in these animals. The results are shown in Table 4.

Evidently, large variations in host size do not influence tumor growth, although in the young, actively growing animals the carcasses maintain their weight better than in the older animals.

In a comparison of tumor growth in males and females of different sizes, data were gathered on tumors which had grown intramuscularly for a longer period of time, i.e., 10 days. In this experiment, likewise, there was no significant difference in tumor size between young and older males or between these animals and adult females (Table 5). The carcass in the actively growing animals is better maintained than in animals which have reached the growth plateau or are showing a reduced rate of growth. However, with a longer

TABLE 2

EFFECT OF SIZE OF INOCULUM ON TUMOR TAKES AND SIZE*

Dilution of tumor suspension	No. rats	No. tumors	Av. wt. of single tumor (gm.)	S. D. tumor wt. (gm.)	Range of tumor wt. (gm.)	No. rats without tumors	No. rats with single tumor
A. Standard tumor cell suspension (100 per cent)	13	26	8.20	±2.77	2.77–13.28	0	0
B. Diluted to 50 per cent	14	27	7.77	±3.08	1.77–14.42	0	1
C. Diluted to 25 per cent	13	20	5.72	±3.04	1.36–11.27	2	2

* 200–250-gm. male albino rats; 0.2 ml. tumor-cell suspension inoculated into flexor muscles of each thigh; 10-day tumor growth.

TABLE 3

COMPARISON OF WALKER TUMOR GROWTH IN ALBINO AND WILD RATS*

	No. rats	No. tumors	Av. initial body wt. (gm.)	Av. final wt. (gm.)	Av. single tumor wt. (gm.)	S. D. (gm.)	Av. wt. adrenal pairs (mg.)
Albino (female)	14	28	234	241	9.30	±1.72	86.8
Wild (6 male+one female)	7	14	272	300	6.58	±3.09	170

* Wild rats fed on dog food, oranges, and given saline to drink; 8 days of tumor growth. Albino rats were tube-fed in this experiment.

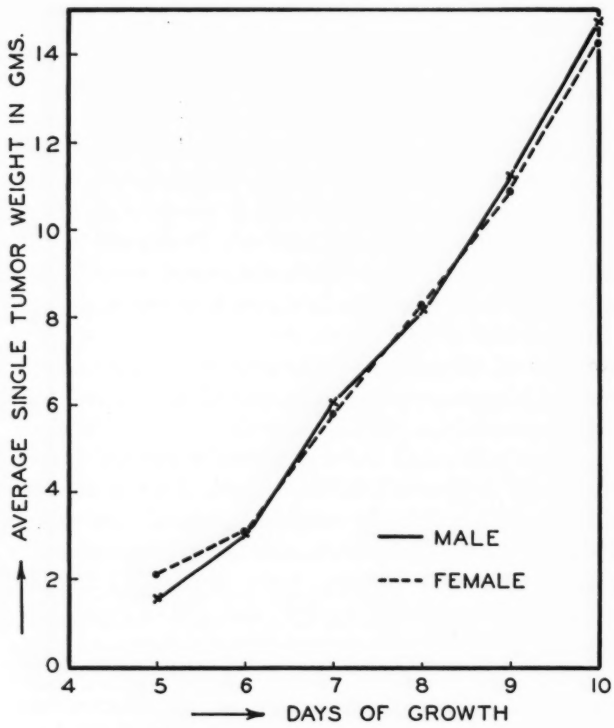

CHART 1.—Comparison of growth rate of intramuscular Walker tumor implants in male and female albino rats.

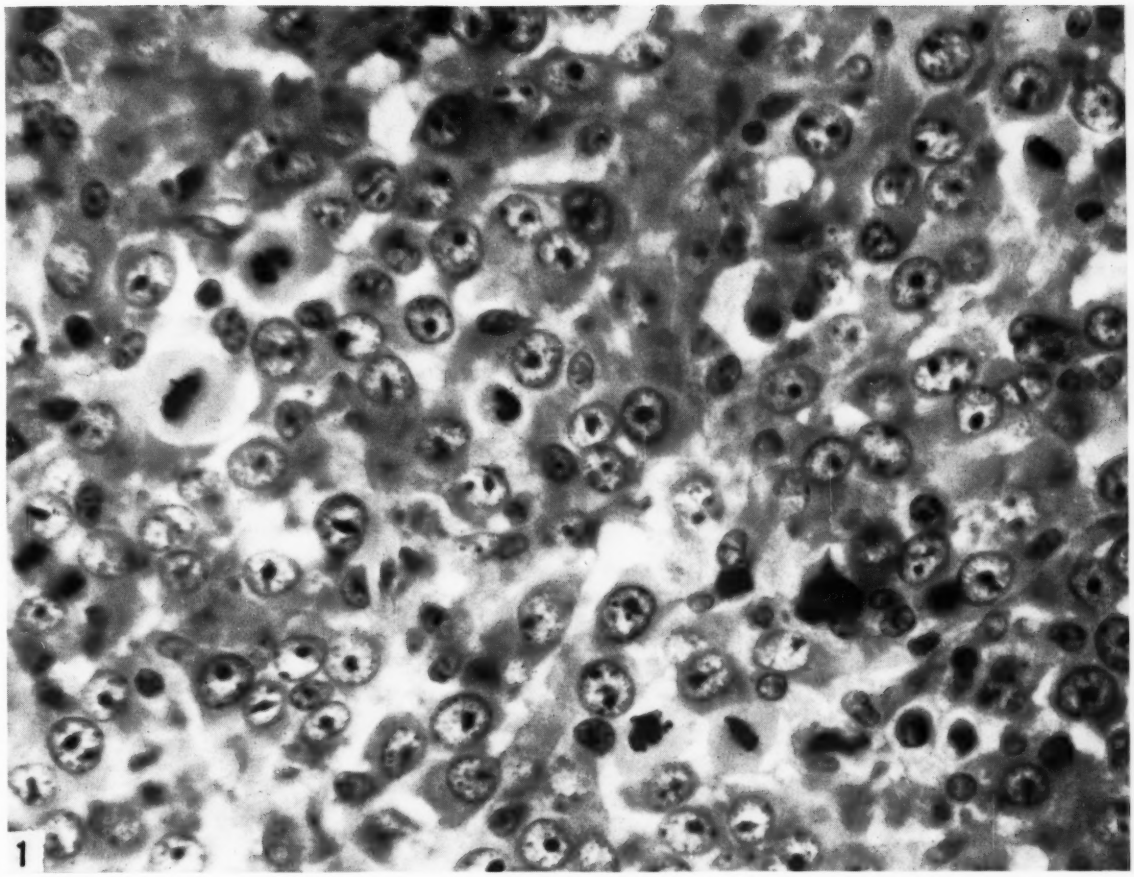

FIG. 1.—Histological appearance of the Walker tumor. Tissue fixed with Zenker-formalin, imbedded in celloidin, and stained with hematoxylin-eosin-azure. Mag. approximately ×1,000.

period of tumor growth the carcasses lose weight rapidly between days 8 and 10 (cf. Table 4 and Table 5).

Neither the sex nor the weight of the animals appears to influence the size of the tumor in a significant manner.

SUMMARY

1. Factors contributing to the variability in growth rate of a transplantable tumor have been analyzed.

2. The Walker tumor was selected because it presents a malignant growth, to which there is 96 per cent susceptibility in either sex of albino rats of the Sprague-Dawley strain. Regressions are extremely rare, and the growth rate is reliable, reproducible, and rapid.

3. The most important factor contributing to variations in growth rate is the site of implantation. Subcutaneous implants show a large variability in size, depending upon whether the tumor becomes vascularized from muscle or skin. Uniform symmetrical growths may be obtained by resorting to bilateral intramuscular implantation into the flexor muscles of the thighs.

4. The growth rate of the tumor is not significantly affected by relatively large changes in the size of the inoculum.

5. In weaned rats, the size and sex of the animal do not influence the growth of the tumor but the carcass weight is greatly affected by the growth phase of the host animal.

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TABLE 4

GROWTH OF INTRAMUSCULAR WALKER TUMOR IN MALE RATS OF DIFFERENT SIZES*

Group	No. rats	Av. initial wt. and range (gm.)	Final total wt. (gm.)	Tumor wt. (gm.)	S. D. tumor wt. (gm.)	Carcass wt. (gm.)	Change in carcass wt. (gm.)
I. Young males	10	178 (169–194)	203	8.66	±2.21	186	+ 8
II. Mature males	10	322 (308–341)	335	10.04	±3.66	315	− 7
III. Old males	10	443 (434–454)	440	9.54	±2.10	421	−22

* Eight days of tumor growth.

TABLE 5

THE GROWTH OF BILATERAL INTRAMUSCULAR WALKER TUMORS IN MALE AND FEMALE RATS OF DIFFERENT SIZES*

Group	No. rats	No. tumors	Av. initial wt. (gm.)	Av. final wt. (gm.)	Tumor wt. (gm.)	S. D. tumor wt. (gm.)	Carcass wt. (gm.)	Change in carcass wt. (gm.)
Growing males	9	18	218	240	14.8	±3.5	210	− 8
Adult females	8	16	252	243	12.9	±2.8	217	−35
Adult males	9	16	343	346	14.5	±3.7	317	−26

* Ten days of tumor growth.

Studies on the Walker Tumor*

II. Effects of Adrenalectomy and Hypophysectomy on Tumor Growth in Tube-fed Rats

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Methods of obtaining standardized non-necrotic growths of the Walker 256 rat tumor suitable for quantitative experimentation have been described in a previous paper (14). The contributions of the adrenal and pituitary glands to the control of growth rate of intramuscular Walker tumor implants are the subject of this paper.

The study of the effects of endocrine extirpations is complicated by the large changes in appetite with resultant dietary deficiencies which may accompany such procedures. In 1914 Rous (12) pointed out the important influence of diet on the growth of some transplantable tumors, but insufficient attention has been paid by most other workers to the control of this factor. In more recent years, Tannenbaum (16) has stressed the profound influence of even moderate caloric restriction on the genesis of spontaneous and induced tumors.

Tumors have been described as efficient "nitrogen traps," (11) in the sense that they not only compete successfully with the carcass for dietary nitrogen, but also can mobilize the substance of the host carcass for their own growth, thus producing the cachexia of advanced malignancy. However, tumor growth is not independent of diet and may be greatly affected by limitations in dietary intake. In a study of the Walker tumor, Walpole (17) demonstrated the retarding effect of progressive starvation on the growth of this tumor, and analysis of his results reveals the severe influence of food restrictions, not only on carcass weight but also on tumor size.

Inherent limitations in the pair-feeding technic have greatly reduced the value of this method of compensating for differences in dietary intake between experimental and control animals. Not only is accurate pair-feeding of large numbers of animals impossible (one is always in arrears), but, when experimental animals have greatly limited dietary intake, the pair-fed controls, which are partially starved, exhibit increased hunger activity and therefore are not normal. The metabolism of an intact animal on a restricted diet may differ markedly from a similar animal, the appetite of which is impaired because of the removal of an endocrine gland.

In an attempt to eliminate the nonspecific effects of partial starvation and to obviate the objections to pair-feeding, we have elected to increase the dietary intake of the experimental group by tube-feeding all our animals, rather than limiting the controls. Tube-feeding is not entirely physiological, but no effect on tumor growth could be demonstrated by tube-feeding intact rats, in comparison to normally fed controls. Feeding by gastric intubation results in accurately known dietary intake of each individual animal and introduces precision and control into biological experimentation.

There is an impressive lack of tumor inhibitory effect of certain relatively toxic compounds when administered to animals in which the carcass weight has been maintained or even increased by tube-feeding adequate amounts of diet. Thus, we have found that 4-dimethylaminostilbene, a toxic but allegedly potent inhibitor of the Walker tumor in rats (5), is essentially without chemotherapeutic effect when administered in similar doses to tube-fed animals. Haddow and his co-workers (3, 5) have studied the profound differences in tumor inhibitory action of a variety of compounds in animals maintained on different levels of protein in the diet.

From a practical point of view, the inhibition of

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tumor growth in malnourished animals is of little interest. *A true chemotherapeutic effect on a tumor can only be said to occur when there is a significant restriction in tumor growth under conditions which promote the well-being of the host.*

REVIEW OF LITERATURE

Alterations of hormonal balance may induce, accelerate, or retard the growth of spontaneous neoplasms. Endocrine effects on transplantable tumors have received relatively little attention.

The relation of the adrenal cortex and its secretions to the growth of tumors has been recently reviewed by Huggins and Bergenstal (6). A number of rodent tumors show depressed growth rate in adrenalectomized animals (4, 6, 8), whereas the growth of certain lymphoid tumors is accelerated by adrenalectomy (13).

Ingle and Baker (8) showed that adrenalectomy in the rat retards the growth of the Murphy-Sturm lymphosarcoma and the Walker tumor. Using tube-fed animals (as well as pair-feeding technic), these investigators demonstrated that adrenalectomy, or adrenalectomy and ovariectomy, performed on the fourth day after implantation of the Walker tumor produced in rats maintained on saline drinking water as substitution therapy a retardation of growth resulting in tumor weights approximately 60 per cent the weight of controls. Hypertrophy of the adrenals has been observed in tumor-bearing animals, and this hypertrophy is dependent upon the presence of the pituitary (2).

McEuen and Thomson (10) studied the effect of hypophysectomy on Walker tumor growth in Wistar rats. In a small series of animals, the tumors grew more slowly in the hypophysectomized group. The hypophysectomized animals never gained weight by amounts exceeding the weight of the tumor. By restricting dietary intake of controls to such an extent as to prevent increase in body weight, these investigators showed that the growth of the tumor was retarded almost as much as by hypophysectomy. They concluded that they were dealing with a nonspecific effect of dietary restriction.

Retardation of growth of a transplanted fibrosarcoma in hypophysectomized rats, compared to that in intact animals, was reported by Loefer (9), but the importance of food control likewise was unfortunately not appreciated in these experiments; the great disparity in the final weights of the two groups makes it impossible to evaluate the role of the pituitary as a factor in the growth of the tumor in the intact controls.

Ball and Samuels (1) re-examined the effect of hypophysectomy on the growth of the Walker tumor in tube-fed male rats, bearing subcutaneous axillary implants of this tumor. These workers fed 1 ml. of a special diet twice daily per 40 grams of body weight to the hypophysectomized rats and restricted the control animals so that the total of tumor and body weight during the experiment remained approximately constant. They concluded that tumor growth is greatly retarded in tube-fed hypophysectomized rats in comparison to controls. The tumor weights of hypophysectomized animals were approximately one-third those of the control group. However, these animals were fed different total amounts; the controls were restricted to less than their normal food consumption, whereas the experimental animals received somewhat more food than they would have voluntarily consumed.

Inhibition of growth of subcutaneous Walker tumor implants by cortisone acetate has been reported by Ingle and coworkers (7), who administered 5 mg. of this steroid daily to 300-gm. tube-fed male rats, beginning on the day following implantation.

METHODS AND MATERIALS

All experiments were performed on Sprague-Dawley (Holtzmann) strain rats. The conditions of maintenance of these animals, the method of implanting the Walker tumor, and the technic of determining tumor size have been described previously (14).

Operations.—The animals were shaved, and all operations were performed aseptically and gently. Bilateral ovariectomy and adrenalectomy were performed through lumbar incisions. Hemostasis was maintained during adrenalectomy by applying silver clips to the adrenal blood supply. Incisions were closed in layers with interrupted silk sutures. Hypophysectomized animals were supplied by Hormone Assay Laboratories, Inc., Chicago, Ill. Completeness of extirpation was determined by an anatomical search for each respective organ at the time of sacrifice, except in the case of hypophysectomized animals, in which the reduction in adrenal and/or gonad weights served as physiologic criteria for the absence of pituitary secretion. Adrenalectomy was performed on hypophysectomized animals at least 1 week after the removal of the pituitary.

Diet.—Stock animals were routinely maintained on standard commercial pellet diet with ample drinking water (14). Hypophysectomized animals received a special diet of bread, buns, oranges, and canned dog food. Adrenalectomized animals received 0.9 per cent aqueous sodium chloride solution for drinking in place of water.

All tumor growth experiments were performed on tube-fed animals. Rats which had been operated upon were allowed to rest for at least 1 week following ovariectomy or adrenalectomy, and for at least 3 weeks following hypophysectomy, or hypophysectomy and adrenalectomy, prior to the institution of tube-feeding.

The tube-feeding of large numbers of rats was greatly facilitated by the use of an automatic diet-dispensing machine, which rapidly and accurately delivers the desired volume of diet. The construction of the machine and technic of feeding are described elsewhere (15).

The semi-liquid diet used in these experiments had the following composition per kilogram: 587 gm. water, 5.0 gm. vitamin mixture,[1] 20.0 gm. Hawkins–Oser modification of Osborne–Mendel salt mixture,[2] 110.0 gm. Armour soluble albumin (containing 85 per cent protein nitrogen), 25.0 gm. Ruffex, 19.0 gm.

[1] The vitamin mixture had the following composition by weight: choline chloride, 30.0; niacin, 0.4; calcium pantothenate, 0.2; pyridoxine hydrochloride, 0.1; riboflavin, 0.2; thiamine hydrochloride, 0.1; and sucrose, 69.0.

[2] The salt mixture had the following composition by weight: calcium carbonate, 6.89; calcium citrate, 30.97; calcium monobasic phosphate, 11.33; magnesium carbonate, 3.34; magnesium sulfate, 3.85; potassium chloride, 12.53; potassium dibasic phosphate, 21.97; sodium chloride, 7.74; and trace elements, 1.38.

The composition of the trace element mixture was: copper sulfate, 0.99; ferric citrate, 92.31; manganese sulfate, 1.15; potassium iodide, 0.29; potassium aluminum sulfate, 0.66; sodium fluoride, 3.61; and zinc chloride, 0.99.

corn oil, 234.0 gm. dextrin, 25 mg. synthetic vitamin K, and 2 drops of oleum percomorphum.

The diet was mixed in 4–6-kg. batches with an Eppenbach turbine mixer. It was stored frozen in plastic containers and warmed to body temperature for feeding.

The majority of rats used in these experiments were females weighing 180–280 gm. They were fed 24 ml. of diet per day, irrespective of body weight. Whereas normal or adrenalectomized animals tolerate two 12-ml. feedings daily, 8 hours apart, an optimal feeding schedule for hypophysectomized rats is 8 ml. every 8 hours. Hypophysectomized animals given three 8-ml. feedings during a 12-hour period each day did not tolerate this procedure well, failed to gain weight, and developed diarrhea. In most of the experiments three daily feedings at 8 A.M., 4 P.M., and 12 midnight were routinely adopted for all animals.

Following the postoperative rest-period, the animals in each experimental series were conditioned to tube-feeding according to the regime outlined in Table 1 until the full diet was well

TABLE 1

SCHEDULE FOR CONDITIONING TO TUBE-FEEDING
(Volume of diet in ml.)

Day	8 A.M.	4 P.M.	6 P.M.	Midnight
1	2		2	
2	3		3	
3	4		4	
4	4	4		4
5	5	5		5
6	6	6		6
7	7	7		7
8 and following	8	8		8

tolerated by the eighth day. A more rapid progression of conditioning is possible for control animals, but those lacking pituitary and adrenals must undergo the more gradual acclimatization. All animals were weighed every 2 days during the experimental period, and implantation of tumor performed only when their weights had stabilized at a level higher than when conditioning was begun. During the period of conditioning, additional rations were permitted and removed only when the full diet was attained by tube-feeding. Tube-fed animals were given ample drinking water.

Cortisone and adrenal cortical extract.—Cortisone acetate was administered as appropriate saline dilutions of commercial Merck "Cortone." This compound was injected subcutaneously into the scruff of the neck once daily. The adrenal cortical extract was an aqueous-alcohol preparation supplied by The Upjohn Co., Kalamazoo, Mich. It was injected intraperitoneally, in doses of 1.5 ml. twice each day approximately 8 hours apart. Injections of cortisone acetate and adrenal cortical extract were begun on the day following tumor implantation and continued until sacrifice of the animal.

RESULTS

Bilateral adrenalectomy and ovariectomy.—The growth of bilateral intramuscular Walker tumor implants in adrenalectomized and adrenalectomized-ovariectomized tube-fed rats maintained on saline drinking water was compared to the growth under similar conditions in sham-operated or intact controls.

In seven consecutive experimental series, involving 86 adrenalectomized and a similar number of control female rats (4–7 months old), Walker tumor growth was retarded in each case in the adrenalectomized group. Eight to 10 days after implantation, the tumors in the adrenalectomized animals averaged 57 per cent by weight of those in the controls (44–68 per cent in different series). (Experiments I–VII of Table 2.) At the same time, the carcass weights (final gross weight minus total tumor weight) of both experimental and control animals approximated the initial weights, not differing in any group by more than ± 6 per cent.

Ovariectomy neither influences the growth rate of the Walker tumor nor does it modify the effect of adrenalectomy. In male rats adrenalectomy likewise retards Walker tumor growth to an extent similar to that observed in female rats. The proportion of successful takes of the Walker tumor is unaffected by adrenalectomy, ovariectomy, or both procedures.

Confirming the results of Ingle and Baker (8), adrenalectomized rats support appreciably slower tumor growth than intact controls, while maintaining carcass weight.

Hypophysectomy.—The growth of intramuscular Walker tumor implants was examined in tube-fed hypophysectomized female rats. These animals were all allowed to rest for at least 3 weeks following surgery, prior to the institution of tube-feeding. During conditioning to tube-feeding, hypophysectomized rats gained rapidly in body weight and, grossly, showed a characteristic obesity lacking in identically fed controls.

Despite the ease with which hypophysectomized rats gained in body weight in response to tube-feeding, they supported much slower growth of the tumor than did control animals. The proportion of successful takes was not affected by hypophysectomy. After 8–10 days of growth, the tumor weights in the hypophysectomized animals averaged 46 per cent of those of the controls (35–63 per cent in different experiments). (Experiments V–VIII of Table 2.)

In four experimental series, each with groups of adrenalectomized and hypophysectomized animals, the removal of the pituitary caused a tumor inhibition similar to that caused by the absence of the adrenals (cf. Experiments V–VII of Table 2). In male rats also hypophysectomy had a retarding effect on tumor growth of the same order of magnitude to that observed in female rats.

Cortisone and adrenal cortical extract.—Cortisone

acetate has been successfully used as total replacement therapy in adrenalectomized man (6), and patients with breast and prostatic cancers have shown significant inhibitions of tumors on such a regime. It became of interest to determine if cortisone acetate could counteract the retarding effect of adrenalectomy or hypophysectomy on the Walker tumor in rats. Large doses of cortisone which produce adrenal atrophy and losses in body weight have been demonstrated to inhibit a variety of transplantable neoplasms (reviewed in [7]).

The effect of small graded doses of cortisone and of aqueous adrenal cortical extract on the Walker tumor was studied in tube-fed adrenalectomized and hypophysectomized rats. The administration of 0.5–1.0 mg. of cortisone acetate daily had essentially no effect on tumor size in intact animals but in each case raised the average tumor size of adrenalectomized or hypophysectomized animals slightly (30–40 per cent). Adrenal cortical extract in doses of 3 ml/rat daily also slightly enhanced tumor growth in adrenalectomized rats (Table 3).

TABLE 2

GROWTH OF WALKER TUMOR IN ADRENALECTOMIZED AND HYPOPHYSECTOMIZED TUBE-FED FEMALE RATS*

Exp. no.	Group	Age (mos.)	No. rats	No. tumors	Days of tumor growth	Av. initial wt. (gm.)	Av. final wt. (gm.)	Av. total tumor wt. (gm.)	Av. single tumor wt. (gm.)	S. D. of single tumor wt. (gm.)	Av. change in body wt. (gm.)	Av. change in carcass wt. (gm.)	Av. paired adrenal wt. (mg.)	Tumor wt. as per cent of growth
I	Adren.	7	11	22	8	276	293	12.44	6.22	±1.84	+17	+ 5		68
	Control (Sham)	7	12	24	8	268	280	18.22	9.11	±2.58	+12	− 6		100
II	Adren.	4–5	10	20	9	267	269	14.50	7.25	±1.47	+ 2	−12		68
	Control	4–5	12	20	9	271	281	21.40	10.70	±2.97	+10	−11		100
III	Adren.	4	12	24	8	242	256	9.60	4.80	±1.87	+14	+ 4		52
	Control	4	14	28	8	234	241	18.61	9.30	±1.72	+ 7	−12		100
IV	Adren.	5	17	34	9	241	240	6.00	3.00	±0.80	− 1	− 7		44
	Control	5	13	24	9	232	234	13.58	6.79	±1.62	+ 2	−12		100
V	Adren.	4	11	22	8	229	239	7.64	3.82	±0.96	+10	+ 2		51
	Hypo.	4	15	30	8	198	228	6.13	3.07	±1.41	+30	+24	19.6	41
	Control	4	11	22	8	230	244	15.02	7.51	±1.87	+14	− 1	75.2	100
VI	Adren.	4	8	16	8	243	244	7.55	3.78	±1.41	+ 1	− 7		47
	Hypo.	4	13	26	8	215	240	7.46	3.73	±1.12	+25	+18	24.6	47
	Control	4	10	20	8	235	239	15.97	7.98	±2.29	+ 4	−12	88.4	100
VII	Adren.	6	17	34	10	226	216	3.98	1.99	±0.77	−10	−14		64
	Hypo.	6	12	24	10	223	239	3.91	1.95	±0.61	+16	+12	19.1	63
	Control	6	14	27	10	229	227	6.22	3.11	±1.46	− 2	− 8	70.3	100
VIII	Hypo.	5	8	16	9	205	220	4.74	2.37	±0.93	+15	+10	22.3	35
	Control	5	13	26	9	232	234	13.58	6.79	±1.62	+ 2	−12	85.4	100

* Tumor and body weights are expressed in grams as arithmetic means. The initial weight was taken at the time of tumor implantation; the final weight was the gross weight of the carcass and tumors at the time of sacrifice. The carcass weight was obtained by subtracting the total tumor weight in each animal from the final weight.

TABLE 3

EFFECT OF CORTISONE AND ADRENAL CORTICAL EXTRACT ON WALKER TUMOR GROWTH IN ADRENALECTOMIZED TUBE-FED FEMALE RATS

Exp. no.	Group	No. rats	No. tumors	Av. single tumor wt. (gm.)	S. D. of tumor wt. (gm.)	Av. paired adrenal wts. (mg.)
IX	Controls	13	24	6.79	±1.62	83.4
	Intact + 0.5 mg. cortisone daily	15	30	6.22	±1.35	82.3
	Adrenalectomized	17	34	3.00	±0.80	
	Adrenalectomized + 0.5 mg. cortisone daily	17	34	3.98	±0.84	
X	Controls	14	28	9.30	±1.72	86.8
	Intact + 3 ml. ACE* daily	16	32	8.98	±1.76	95.7
	Adrenalectomy	12	24	4.80	±1.87	
	Adrenalectomy + 3 ml. ACE daily	14	28	5.88	±1.25	
XI	Controls	10	20	10.70	±2.97	
	Intact + 1.0 mg. cortisone daily	9	18	10.44	±3.30	
	Intact + 2.5 mg. cortisone daily	12	24	10.22	±3.02	
	Adrenalectomy	10	20	7.25	±1.47	
	Adrenalectomy + 1.0 mg. cortisone daily	11	22	8.12	±2.31	
	Adrenalectomy + 2.5 mg. cortisone daily	11	22	8.97	±1.68	

* ACE = Aqueous adrenal cortical extract (biological potency reported to be such that 1 ml. = 0.1 mg. of compound F in the rat liver glycogen deposition test).

These small doses of cortisone or adrenal cortical extract do not induce adrenal atrophy, nor do they cause the animals to lose weight. It was therefore not possible to substitute completely for the presence of the adrenal in as far as tumor growth is concerned with cortisone or aqueous adrenal cortical extract in any of the doses used.

Hypophysectomy and adrenalectomy.—Since the presence of the adrenal glands and of the pituitary is necessary for maximal growth of the Walker tumor, the question arose whether the effects of hypophysectomy were caused solely by secondary adrenal insufficiency. There is evidence that the hypophysectomized rat has residual adrenal cortical function. The most direct approach to the evaluation of the individual contributions of the adrenal and the pituitary to tumor growth appeared to be through the use of adrenalectomized-hypophysectomized rats.

Albino rats tolerate adrenalectomy as early as 1 week following hypophysectomy and may be maintained on saline as substitution therapy. These animals did not tolerate tube-feeding beyond 15 ml diet/day. This difficulty is apparently one of combined adrenal and pituitary insufficiency, since rats subjected to either procedure alone tolerate tube-feeding well.

Since it was necessary to feed full amounts of diet to increase carcass weights, it was found that even very small doses of cortisone permitted adrenalectomized-hypophysectomized animals to be tube-fed and to gain weight under these conditions. The doses of cortisone involved, 0.25–0.50 mg/rat/day, do not cause adrenal atrophy in intact rats, but do have a slight tendency to increase tumor weights in adrenalectomized or hypophysectomized animals.

Table 4 represents an experiment in which intact, adrenalectomized, hypophysectomized, and adrenalectomized-hypophysectomized rats were tube-fed, with and without 0.5 mg. cortisone acetate daily. The average tumor weight of the adrenalectomized-hypophysectomized group was 1.65 ± 0.70 gm., whereas the control tumor weight was 6.22 ± 1.35 gm., a ratio of almost 1:4.

TABLE 4

GROWTH OF WALKER TUMOR IN ADRENALECTOMIZED, HYPOPHYSECTOMIZED, AND HYPOPHYSECTOMIZED-ADRENALECTOMIZED TUBE-FED FEMALE RATS WITH CORTISONE SUPPLEMENT*

Group	No. rats	No. tumors	Av. initial wt. (gm.)	Av. final wt. (gm.)	Av. total tumor wt. (gm.)	Av. single tumor wt. (gm.)	S. D. of single tumor wt. (gm.)	Av. change in body wt. (gm.)	Av. change in carcass wt. (gm.)	Av. paired adrenal wt. (mg.)	Tumor wt. as per cent of controls	Tumor wt. as per cent of intact + 0.5 mg. cortisone
Adren.	17	34	241	240	6.00	3.00	±0.80	− 1	− 7		44	
Adren. + 0.5 mg. cort. daily	17	34	239	231	7.96	3.98	±0.84	− 8	−16		59	64
Hypo	8	16	205	220	4.75	2.37	±0.93	+15	+10	21.2	35	
Hypo + 0.5 mg. cort. daily	13	26	215	225	5.83	2.92	±0.97	+10	+ 4	21.3	43	47
Hypo-Adren. + 0.5 mg. cort. daily	16	32	180	191	3.29	1.65	±0.70	+11	+ 8		24	27
Intact + 0.5 mg. cort. daily	15	30	241	241	12.43	6.22	±1.35	0	−12	82.3	92	100
Controls	13	26	232	234	13.23	6.79	±1.62	+ 2	−11	85.4	100	

* The age of the rats was 5 months; the duration of tumor growth 9 days.

DISCUSSION

Under standardized conditions, intramuscular Walker tumor implant growth is markedly inhibited by the absence of the pituitary or the adrenals in tube-fed animals in which carcass weight is maintained or increased. The combined removal of both endocrine organs causes a cumulative restriction of tumor growth. Adrenalectomy inhibits the tumor size to *ca.* 57 per cent of controls, and hypophysectomy restricts growth to *ca.* 46 per cent of controls. A convenient graphical method of demonstrating the differential effect on tumor and on carcass weights is shown for a typical experiment in Chart 1. The effect of adrenalectomy cannot be counteracted by the administration of cortisone acetate or aqueous adrenal cortical extract. Combined adrenalectomy and hypophysectomy in tube-fed animals given small doses of cortisone (which cause neither weight loss nor adrenal atrophy) produces a further restriction of tumor growth rate to some 25 per cent of those of controls. These endocrine extirpations do not affect the high incidence of successful takes of the Walker tumor in the rat.

In the absence of a better understanding of the mechanism by which the pituitary and adrenals regulate tumor growth, it would be premature to ascribe these effects to the direct influence of specific, unknown hormones on tumor metabolism.

The possibility remains that the inhibition of tumor growth may be dependent upon indirect factors, such as vascular supply and connective tissue reaction.

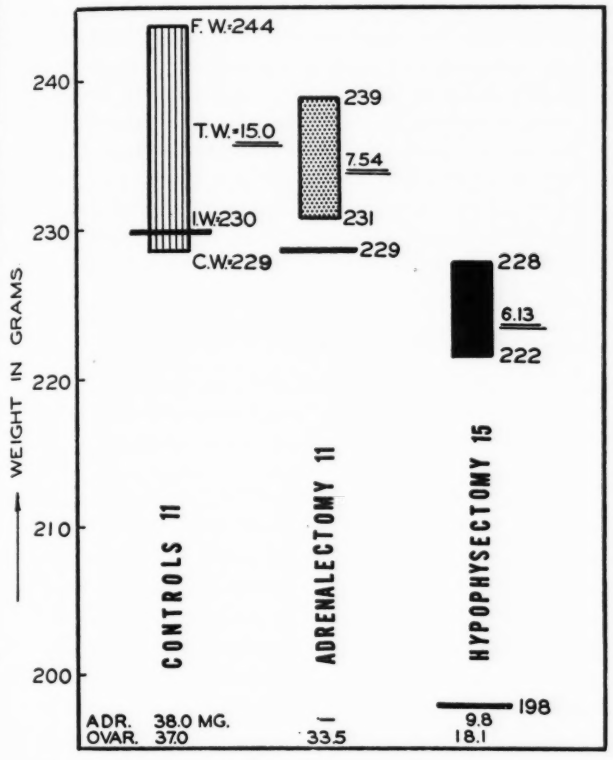

CHART 1.—Effect of adrenalectomy or hypophysectomy on intramuscular Walker tumor growth in 8 days in tube-fed female rats. The heavy horizontal lines (*I.W.*) represent the average initial weight of each group of animals at time of tumor implantation. The upper limit of each shaded area is the average final group weight (*F.W.*) at the time of sacrifice, and the lower limits represent the carcass weights (*C.W.*). The tumor weight (*T.W.*) represented by the shaded area is the total weight of both tumors. The number after each group name is the total number of animals. At the bottom are given the average paired weights of the ovaries and the adrenals in milligrams.

It may be concluded that the adrenals and pituitary are essential for maximal growth of the Walker tumor and that the pituitary exerts its action on the tumor to a large degree independently of the pituitary-adrenal axis.

SUMMARY

1. Bilateral adrenalectomy in tube-fed rats maintained with saline drinking water inhibits the growth of intramuscular Walker tumor implants to *ca.* 57 per cent of controls.

2. Hypophysectomy in tube-fed rats inhibits the growth of intramuscular Walker tumor implants to *ca.* 46 per cent of controls.

3. Cortisone acetate or adrenal cortical extract in doses used can only to a slight extent counteract the absence of the adrenals or pituitary.

4. Combined removal of adrenals and pituitary results in a cumulative restraint on tumor growth.

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Book Reviews

Theoretische Biologie. 2d Vol. Stoffwechsel, Wachstum. By Ludwig von Bertalanffy. Bern, Switzerland: A. Francke Verlag, 1951. Pp. 418. S. Fr. 47.50.

In these overspecialized times of ours it is highly desirable to be presented with a book whose author tries to give a synthesis of the vast material collected in the scientific laboratories. This book is apparently not intended for the specialist, but for those research workers in biology and related fields who wish to keep in touch with the over-all problems of biologic research. It seems almost impossible for one man alone to cover both fields: growth and also metabolism. One gets the impression that the first part—dealing with enzymes and metabolism—is not the author's domain. So it might be understandable that the chapters covering coenzymes of hydrogen transport and oxidative phosphorylation are not up to date as far as their content is concerned, and they are even occasionally misleading in their conclusions.

The second part of this monograph offers a good survey on growth and its manifold relations to biologic problems. The question of growth is treated for both unicellular and multicellular organisms and populations. Here, the author presents a great deal of his own work and experience.

Hanns Schmitz

McArdle Memorial Laboratory
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Announcements

The American Association for Cancer Research

ANNUAL MEETING

The forty-fourth annual meeting of the American Association for Cancer Research, Inc., will be held in Chicago on April 9–11, 1953, in the Drake and Knickerbocker Hotels.

Abstracts of papers accepted for presentation at the meeting will be published and distributed to members of the Association before the meeting as "Proceedings of the American Association for Cancer Research," which will replace the April issue of *Cancer Research*. Copies of the Proceedings also will be available for sale at the time of the meeting.

The following regulations concerning abstracts and presentation of papers have been established by the Program Committee (Drs. G. O. Gey, A. Cantarow, and G. B. Mider) and approved by the Board of Directors of the Association. Failure to comply with the requirements will result in the rejection of abstracts. Papers represented by rejected abstracts will not be considered by the Program Committee, and such abstracts will not be published in the Proceedings. No abstract of a paper accepted by the Program Committee, either for oral presentation at the meeting or for presentation by title, can be withdrawn from publication or altered in composition by the author.

a) Four copies of the abstract are to be sent to the chairman of the Program Committee, Dr. George O. Gey, Department of Surgery, The Johns Hopkins Hospital, Baltimore 5, Md.

b) The abstracts are required by *January 3, 1953*, and must be postmarked not later than *December 30, 1952*.

c) A member of the Association may submit only one paper of which he is the principal author and one paper of which he is the co-author. This rule also applies to a nonmember. Abstracts from nonmembers will be considered only if introduced by a member. Authors may request that their paper be "read by title." Such requests will be recorded on the printed program.

d) Abstracts must adequately describe the objectives and results of the research, so that the committee may be able to judge the quality, originality, and completeness of the work. Authors must accept sole responsibility for their statements and should exercise great care in the preparation of the abstracts; galley proof cannot be sent because of time limitations.

e) A request for presentation of a paper at the meeting of the American Association for Cancer Research, Inc., implies that the author has not presented, or is not planning to present, the same subject matter at a meeting of another national scientific organization.

f) Abstracts must not be longer than 250 words. The style used in previous abstracts (see *Cancer Research*, April, 1952) is to be followed. The title of the paper is to be followed by the names of the authors, their university, laboratory or hospital, and its location. *The names of nonmembers should be marked with an asterisk; when needed, the name of the member introducing the paper is to be included, i.e. "(Introduced by A. J. Dee)."* Abstracts must be typewritten, double-spaced, on $8\frac{1}{2} \times 11$-inch paper.

g) If reprints are desired, the requests must accompany the original abstracts.

The time allowed for presentation of any paper will be 10 minutes. Projectors will be provided only for lantern slides $3\frac{1}{4} \times 4$ inches or 2×2 inches.

By action of the Members of the Association, a registration fee of $3.00 will be charged each member or nonmember attending the scientific sessions.

Hugh J. Creech
Secretary-Treasurer
American Association for Cancer Research
Institute for Cancer Research
Fox Chase, Philadelphia 11, Pa.